# THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

ARTHUR HARDEN

AND

CHARLES ROBERT HARINGTON

EDITORIAL COMMITTEE

PROF. G. BARGER
PROF. V. H. BLACKMAN
MR J. A. GARDNER
SIR F. G. HOPKINS
SIR F. KEEBLE
PROF. W. RAMSDEN
SIR E. J. RUSSELL

VOLUME XXVIII 1934

PART II, pp. 1149 to end

CAMBRIDGE
AT THE UNIVERSITY PRESS
1934

Vol. XXVIII, No. 4 1934

MEDICAL LIBRARY OCT 24 1934

# THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

ARTHUR HARDEN

AND

CHARLES ROBERT HARINGTON

EDITORIAL COMMITTEE

PROF. G. BARGER
PROF. V. H. BLACKMAN
MR J. A. GARDNER
SIR F. G. HOPKINS
SIR F. KEEBLE
PROF. W. RAMSDEN
SIR E. J. RUSSELL

LONDON:
CAMBRIDGE UNIVERSITY PRESS
Fetter Lane, E.C. 4
also
CHICAGO: The University of Chicago Press
(Agent for the United States)
BOMBAY, CALCUTTA, MADRAS: Macmillan
TOKYO: Maruzen Company, Ltd.

*All rights reserved*

1934

*Price Fifteen Shillings net*

PRINTED IN GREAT BRITAIN

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted solely for the advancement of the Science of Biochemistry. Persons interested in Biochemistry are eligible for election.

Meetings are held at different centres for the communication of papers to the Society.

Members are entitled to receive a copy of the Journal. Further information as to the conditions of membership may be obtained on application to the Hon. Sec. Prof. A. C. Chibnall, Imperial College of Science and Technology, South Kensington, S.W. 7, or to the Hon. Treas., Mr J. Addyman Gardner, 24 Palace Mansions, Kensington, W. 14, to whom subscriptions should be sent.

Papers for publication should be sent to Prof. A. Harden, F.R.S., Sunnyholme, Bourne End, Bucks. Communications respecting the printing of the articles, or respecting the purchase of offprints should be addressed to the University Press, Cambridge.

The Journal is issued about every two months and the date at which each paper is received by the editors is printed at the beginning of the paper.

All communications respecting the purchase of copies of the parts or volumes, whether current or back issues, or respecting subscriptions in the case of non-members of the Biochemical Society (£3. 10*s*. 0*d*. net per volume (post free) payable in advance) should be addressed to The Cambridge University Press, Fetter Lane, London, E.C.4, or to the Hon. Treas., Mr J. Addyman Gardner, 24 Palace Mansions, Kensington, W. 14. For prices of back numbers and volumes see list on p. 4 of Wrapper.

Quotations can be given for Buckram binding cases and for binding Subscribers' Sets.

The Cambridge University Press has appointed the University of Chicago Press agent for the sale of the *Biochemical Journal* in the United States of America.

## Notice to Contributors.

1. Contributors who are members of the Biochemical Society receive 25 copies (without covers) of their papers free and can purchase additional copies if notice be given when their proofs are returned. Other contributors may purchase reprints but receive no free copies.

2. Papers forwarded to the Editors for publication are understood not to be offered to any other Journal for prior or simultaneous publication.

3. All communications intended for publication must be type-written.

4. Proofs are issued in final paged form and no proof corrections will be accepted which involve change in the paging.

5. Contributors residing outside Europe are requested to nominate someone resident in Great Britain to be responsible for the correction of the proofs of their papers. Failing this the proofs will be corrected by the Editors. Papers from such contributors should be accompanied by a statement of the number of reprints required.

6. **References.** References to literature should be arranged alphabetically according to authors' names at the end of the communication, each accompanied by the date, title of Journal, volume and page, thus: Ackermann (1912), *Z. Biol.* 59, 17. In the text the reference should be indicated by the author's name and the date, enclosed in brackets, thus [Ackermann, 1912]. If reference is made to several papers published by one author in a single year, these should be numbered in sequence and the number quoted along with the year both in the text and in the collected references.

7. Chemical formulae should be written, as far as possible, in a single horizontal line.

8. Illustrations and curves accompanying the papers must be carefully drawn about twice the size of the finished block, on smooth white Bristol boards or ordinary curve paper in Indian ink. Any lettering on these drawings should be lightly inserted in pencil. Further information can be obtained from the Editors.

MSS. for the successive numbers of Vol. 28 should be in the hands of the Editors before the 1st of January, March, May, July, September and November respectively. Contributors are specially requested to arrange references in the manner uniformly adopted in this Journal.

# OBITUARY NOTICE.

## WILLIAM BATE HARDY (1864–1933).

WILLIAM BATE HARDY'S career showed how a man of genius can attain to high success in activities quite other than those with which his early training was concerned. He was a biologist whose work greatly advanced physical chemistry, and a University don who became an administrator ably controlling an important and extensive national enterprise. At the same time it is easy to see that there was complete continuity and a logical sequence in the development of his own thought and work.

Hardy was educated at Framlingham and at Gonville and Caius College, Cambridge. In 1888 he obtained a First Class in Part II of the Natural Sciences Tripos being one of a group of seven, of whom two others besides himself were to become Fellows of the Royal Society, namely, the late Hugh Kerr Anderson, Master of Gonville and Caius, and Arthur Hutchinson, the present Master of Pembroke College, Cambridge. Hardy's subject was zoology.

After graduation he was elected to a Shuttleworth Research Scholarship and in 1900 was Thurston Prizeman. In 1892 he became Fellow of his College and later Tutor. In the University he was first Demonstrator and later Lecturer in Physiology.

While a member of Michael Foster's Staff he was more particularly concerned with the teaching of histology to the advanced class. He turned to research in that subject and between 1892 and 1898 he published eleven papers on the morphology, behaviour and functions of the leucocytes or wandering cells of crustacea, amphibia and mammals. In these studies he displayed great technical skill. It was a time when special interest was felt in leucocytes as the result of Metchnikoff's discovery of phagocytosis. Hardy contributed much to the knowledge of that phenomenon, and among many facts of interest he demonstrated the existence of the remarkable "explosive" corpuscles of crustacean blood.

Towards the end of this period of his activity he had become convinced that the current technique of histology required critical reconsideration. The methods whereby living tissues were "fixed" by reagents for microscopical examination had for some years served a useful purpose, but they had been often misleading, replacing real by artificial appearances in the cell protoplasm. "Reagents" wrote Hardy, "confer a structure on colloidal matter which differs in most cases in kind and in some cases in degree from the initial structure. Hence it is inferred that the structures seen in cells after fixation are due, to an unknown extent, to the action of fixing reagents."

Convinced of the fundamental importance of such considerations to cytology and to biology in general, Hardy began with characteristic energy to study for himself the effect of associated conditions on the stability and behaviour of colloidal systems. He worked with little previous acquaintance with physical chemistry and with the simplest equipment, but quickly obtained new data of prime importance. The two classical papers which he published in 1899 [*J. Physiol.* **24**] "On the structure of cell protoplasm" and "On the coagulation of proteid by electricity" exerted a great influence upon the thought of the time. Together they covered a very wide ground. As the result of a year's work



Vol. 28, p. 1149, under Obituary Notice
*for* (1864–1933) *read* (1864–1934)

Hardy had made much clearer than it had been before the distinction between suspensoid and emulsoid colloidal systems and demonstrated many important properties of each type. His careful study of the coagulating power of electrolytes, showing its dependence upon micellar and ionic charges, gave precision to knowledge previously vague and led to much later research by himself and many others, and to fundamental advances in knowledge. These pioneer papers were soon followed by others developing the subject; one for instance dealing with the conditions which determine the stability of irreversible hydrosols, and another on the mechanism of gelation in reversible colloid systems [*Proc. Roy. Soc. Lond.* (1900), **66**].

For a short interlude at this time he concerned himself with the effect of radium upon living tissues and was one of the earliest to study the phenomena involved. The danger of handling radium was then not fully recognised, and it is perhaps well that he did not long continue this work, for he was certainly careless in his dealings with the not inconsiderable amount he possessed. He received a severe burn as the result of carrying it in his waistcoat pocket.

Hardy early displayed an interest in the equilibrium of proteins in the blood and retained it to the end of his life. He always tended to think that in the circulating blood some unitary protein-complex exists, but he studied the recognised fractions and the globulin fraction in great detail. The work upon globulins published in 1903 and 1905 [*J. Physiol.* **29** and **33**] was summarised in his Croonian Lecture in 1907. It led to several generalisations important to colloid science, among which was the establishment of the isoelectric point as a physical constant for individual proteins.

At this time he became immersed in problems of molecular physics in so far as they bore upon his own work, and to qualify for dealing with them he set himself to refresh and extend his knowledge of mathematics. In the years just before the war he published papers in which mathematical analysis was applied to phenomena which he had previously studied more qualitatively. Such were the following: "The general theory of colloidal solutions" [*Proc. Roy. Soc. Lond.* **86**]; "The tension of composite fluid surfaces" [*Proc. Roy. Soc. Lond.* **86** and **88**]; and "An application of the principle of dynamical similitude to molecular physics." These theoretical papers are important as showing the working of their author's mind at this time, but most find them difficult to understand and they have not counted for so much as the publications in which Hardy has described experimental work of his own. The period of their appearance was one of self discipline in which he was preparing for later important work.

Immediately after the war his interest turned to the phenomena associated with static friction and thence inevitably to the problems of lubrication. Such subjects might seem remote from his earlier interests, but as a matter of fact they followed from them in due sequence. He had long held that "colloidal solutions are merely special cases of ordinary molecular solution which are peculiar solely in a wide departure from thermodynamic reversibility due to the operation of a large internal frictional constraint which springs from the limited mobility of one of the constituents." Though it is a far cry from such "frictional constraint" to the friction between solid surfaces Hardy's thought about the one led to his interest in the other.

His two papers on static friction [*Phil. Mag.* (1919), **38**; and (1920), **39**] describe experiments of great interest and discuss from interesting standpoints the relations established between solid surfaces when in contact. The later of these papers gives the results of his remarkable study of the relation between

the molecular constitution of substances and their activity as lubricants. This work was summarised in an Evening Address delivered at the Royal Institution in February 1920. The subject of boundary lubrication was later developed in other papers. One, published with Miss Ida Doubleday, dealing with "The latent period and mixtures of two lubricants" is of special interest as it contains one of the earliest references to the orientation of molecules at a solid surface. Hardy at this time recognised to the full the importance to surface orientation of the groups we now speak of as polar. This work was summarised and on the theoretical side extended in the Bakerian Lecture for 1925 [with I. Bircumshaw; *Proc. Roy. Soc. Lond.* **108**].

The study of boundary lubrication was next extended to the subject of Adhesion, to which—working and publishing conjointly with Miss Millicent Nottage—he contributed much that was original [*Proc. Roy. Soc. Lond.* A **112**, **118**]. Hardy was impressed by the evidence which convinced him that molecular orientation induced by surface forces may be transmitted through layers a thousand molecules in thickness, and as he never forgot the biological bearings—actual or possible—of all that he did in physical chemistry or molecular physics, he was tempted to see in such transmitted orientation a mechanism for the maintenance of static muscular tonus by the central nervous system [*J. Gen. Physiol.* (1927), **8**].

Among his latest scientific papers was an important one on "Problems of the boundary state" published in the *Philosophic Transactions of the Royal Society* for 1931; and one with the title "Absorption: A study of availability and accessibility [with M. E. Nottage, *Proc. Roy. Soc. Lond.* A **138**].

By this time he had become a recognised authority on the subject of boundary conditions, and he contributed an article on the subject to the *Dictionary of Applied Physics*. His pioneer work undoubtedly greatly helped to stimulate modern research on the properties of films and on surface chemistry in general.

Hardy's personal thought always ran on the lines of physics and physical chemistry rather than on those of organic chemistry, and in his earlier days at least he showed no special interest in the chemical reactions of cell metabolism, or indeed in metabolic problems generally. In 1915, however, he became Biological Secretary of the Royal Society at a time when the problems of national nutrition were very urgent and when it was felt that the Society should lend scientific aid towards their solution. As secretary, Hardy organised the "Food (War) Committee," and during the succeeding years while that committee was at work he came into close touch with the many problems of food values, food supplies and food transport. He became keen upon, and well versed in, such matters, and his grasp of them was recognised by his appointment as first Chairman of the Food Investigation Board and Director of Food Investigation in the Department of Scientific and Industrial Research. In such posts he found a new field for the exercise of his powers. He became responsible for the official research work carried out at three important stations—the Low Temperature Research Station at Cambridge, the Torry Research Station and the Ditton Laboratory, and for years he spent an immense amount of time on constructive work at a variety of committees. In all these activities his success was outstanding, gaining for him the confidence of administrators and industrialists in equal measure. Marine research also occupied much of his time in his later days. For nine years he was Chairman of a Committee appointed by the Development Commissioners to advise on fishery investigations, a post for which he was highly qualified both by his biological knowledge and his life-long knowledge of the sea in all its moods.

In the midst of his administrative labours he retained all his interest in pure science, as his latest published papers show. He stood firm moreover in his belief that *ad hoc* technical researches must be based upon the results of fundamental research, and those who conducted the former under his guidance were encouraged at the right moments to pursue the latter.

In addition to holding the important offices already mentioned he was a member of the Economic Advisory Council, President of the British Association of Refrigeration, a Trustee of the National Portrait Gallery and a member of the Governing Body of Charterhouse and of the Leverhulme Trust Committee. He received many honours. He was Royal Medallist, and, as already mentioned, Croonian and Bakerian Lecturer of the Royal Society. He received honorary degrees from Oxford, Aberdeen, Birmingham and Edinburgh. In 1931 he delivered the Abraham Flexner Lectures at Vanderbilt Univerity. He was knighted in 1925. At the time of his death he was President of the British Association and it was a matter of profound regret not only for his many friends in Aberdeen where the Association meets this year, but to every member of the Association that he did not live to deliver his presidential address.

Hardy's whole personality was strong and vital. He approached every task with enthusiasm and zest and enjoyed life's better kinds of pleasure with an equal zest. For him the greatest of these pleasures—as all his friends knew—was salt-water sailing. Those who shared his voyages in his younger days delighted to tell of the tight places his love of adventure took them into, and of the skill and resourcefulness which always brought them safely out. He loved Nature in the wild and he loved good literature. He greatly valued the gifts of friendship and repaid them in full measure from his very warm heart.

He married in 1898 Alice Mary the daughter of Mr G. B. Finch who survives him with his son and two daughters.

F. G. H.

# CLIV. THE CHEMICAL DETERMINATION OF VITAMIN C WITH REMOVAL OF INTERFERING REDUCING AND COLOURED SUBSTANCES.

By ADRIANUS EMMERIE and MARIE VAN EEKELEN.

*From the Laboratory of Hygiene, The University of Utrecht.*

(*Received May 11th, 1934.*)

Birch *et al.* [1933] have demonstrated that the titration of ascorbic acid with 2:6-dichlorophenolindophenol must take place in acid solution, because substances like glutathione and ferrous salts, which reduce the indicator in neutral medium, do not show this property in acid solution.

Tillmans *et al.* [1932] have pointed out the fact that it is necessary to reduce with $H_2S$, because ascorbic acid can also be present in the reversibly oxidised state.

We have found that ascorbic acid is not stable in dilute trichloroacetic acid solutions, even in the presence of $H_2S$.

Table I.

Ascorbic acid solution 2 ml. = 0·25 ml. indicator. In different concentrations of trichloroacetic acid under $H_2S$ for 16 hours.

| Trichloroacetic acid concentration % | ml. indicator per 2 ml. solution |
|---|---|
| 3 | 0·15 |
| 1·5 | 0·23 |
| 0·75 | 0·24 |

However, in acetic acid solutions, ascorbic acid in the presence of $H_2S$ is very stable. In different concentrations of acetic acid up to 10 % under $H_2S$ after 18 hours no difference in titration value was found.

In a previous communication one of us [Emmerie, 1934] has demonstrated that cysteine and probably other unknown substances, which reduce the indicator in acid solution, can be precipitated by mercuric acetate. The ascorbic acid is not precipitated but remains in solution in the reversibly oxidised state. After centrifuging, the solution is treated with $H_2S$ to reduce the oxidised form and to remove the excess of mercury which is precipitated as HgS.

Precipitation with mercuric acetate has also proved to be very important in relation to red-coloured plant juices or extracts (*e.g.* red cabbage and tomato), because it is impossible to titrate these coloured solutions with the indicator, whereas the colour is removed by precipitation with mercuric acetate.

We give below a description of our technique for determining the ascorbic acid content of different biological fluids and tissue or plant extracts.

## Experimental.

*Blood.* To 10 ml. newly shed oxalated blood (in an Erlenmeyer flask of 100 ml.) 10 ml. 10 % trichloroacetic acid are added. After thorough mixing and precipitation of the proteins 5 ml. 20 % mercuric acetate solution are added and well mixed by stirring the contents of the flask. After some minutes 0·5 g. of pure $CaCO_3$ is added to neutralise the trichloroacetic acid. The mixture must be shaken till a drop gives a faint violet colour with a strip of Congo red paper. The mixture is centrifuged, and the supernatant liquid is decanted off and treated with $H_2S$. After precipitation of the HgS, the solution is filtered and left overnight with $H_2S$. The $H_2S$ is removed by a stream of pure nitrogen (controlled by lead acetate paper). This removal is complete after about 10–30 minutes. For titration 5 ml. of the solution are taken, to which is added 1 ml. 10 % trichloroacetic acid.

5 ml. filtrate from human blood use about 0·1–0·25 ml. indicator (1 mg. ascorbic acid = 12 ml. indicator).

When a definite quantity of pure ascorbic acid was added to the blood, this quantity was completely recovered by this procedure.

*Urine.* To 20 ml. fresh urine diluted 1 : 6 are added 10 ml. 20 % mercuric acetate solution. After centrifuging, the supernatant liquid is decanted off and treated with $H_2S$. The further procedure is the same as for blood. In normal cases 5 ml. filtrate are titrated to which 1 ml. 10 % trichloroacetic acid is added.

*Milk.* 25 ml. milk are mixed with 15 ml. 20 % mercuric acetate solution and the same procedure as given for urine is followed. 5 ml. filtrate are titrated.

*Tissue and plant extracts.* The material is ground up with iron-free pure sand in a mortar with 3 % trichloroacetic acid solution. The quantity of trichloroacetic acid solution is about 5–10 times the weight of the tissue or plant material. After centrifuging, the extract is neutralised with $CaCO_3$ and filtered. To a known volume of this extract mercuric acetate solution is added till no further precipitation takes place. After centrifuging the procedure as given above is followed.

## Summary.

Methods are given for determining the ascorbic acid content of blood, urine, milk, tissue and plant extracts with removal of interfering reducing and coloured substances by means of mercuric acetate.

## References.

Birch, Harris and Ray (1933). *Biochem. J.* **27**, 580, 590.
Emmerie (1934). *Biochem. J.* **28**, 268.
Tillmans, Hirsch and Dick (1932). *Z. Unters. Lebensm.* **63**, 267.

# CLV. ARGINASE AND ARCAIN.

By ERNEST BALDWIN[1].

*From the Biochemical Laboratory, Cambridge.*

(*Received May 19th, 1934.*)

INTEREST in the enzyme arginase has recently been considerably stimulated, firstly by the discovery of argininephosphoric acid in a number of invertebrates [Meyerhof and Lohmann, 1928; Needham *et al.*, 1932], and secondly by the realisation of its importance in the elaboration of urea [Krebs and Henseleit, 1932]. A survey of the invertebrate phyla is now in progress in this laboratory with a view to ascertaining the distribution of arginase.

Arnold and Luck [1933] have already examined many invertebrates for the presence of arginine, since such a study is of the greatest importance for the comparative biochemistry of muscle. Their method [Kiech *et al.*, 1931] assumes the perfect specificity of arginase. Tissue extracts are submitted to the action of that enzyme, urea being then isolated as dixanthydrylurea, followed by a volumetric estimation of that compound. In several respects their results appear to conflict with those of other authors; thus the polychaete worms, represented by *Nereis*, were found to contain no arginine, whereas Needham *et al.* [1932] demonstrated the presence of a phosphagen which showed the behaviour typical of argininephosphoric acid. Again, the cephalopoda proved to contain arginine in considerable quantity, whereas Baldwin [1933] has shown that in all probability the cephalopod phosphagen is not argininephosphoric acid. The possibility that other phosphagens may be present in the invertebrate phyla has, in the author's opinion, not been investigated with sufficient thoroughness. The harmonisation of certain of Arnold and Luck's results with those of Needham *et al.* [1932] must depend largely upon such studies.

Of special interest in this connection is the lamellibranch class of the Mollusca, for here very variable results have been obtained by various workers. Meyerhof [1928] found argininephosphoric acid in *Pecten* but could detect no labile phosphate whatever in *Pinna*, *Cytherea* or *Anodonta*, while Borsuk *et al.* [1933] have confirmed Meyerhof's findings with regard to *Pecten*. Arnold and Luck [1933] report that among nine such molluscs examined by them arginine was found in every case. The lack of concordance between the distribution of arginine itself and that of its phosphoric acid compound suggested that some other compound might be involved, and arcain, the substance recently isolated from *Arca noae* by Kutscher, Ackermann and Flössner [1931], at once suggested itself, particularly since it appears to be of random distribution. Thus it is present in *Arca noae* but absent from *Mytilus* [Kutscher and Ackerman, 1931]. Arcain has been shown by Kutscher, Ackermann and Hoppe-Seyler [1931] to be identical with tetramethylenediguanidine, which was first synthesised by Kiesel [1921, 1] and found by him to be attacked by a preparation of *Aspergillus* arginase, giving urea and agmatine [1921, 2].

It therefore seemed possible that arcain might also be attacked by animal arginase, and would in this case interfere with the estimation of arginine, since

[1] Senior 1851 Student.

it too would give urea on hydrolysis. Arnold and Luck [1933], Meyerhof [1928], Baldwin and Needham [1933] and many more have used arginase as a specific test for the presence of arginine, for its specificity appeared to be absolute. Even $\gamma$-guanidinobutyric acid, which was formerly believed to be attacked [Thomas, 1913] has since been shown not to be affected [Thomas *et al.*, 1922], and repeated observations with many possible substrates have only confirmed the apparently complete specificity of arginase [Haldane, 1930]. Nevertheless it cannot be too greatly stressed that material of practically unknown composition, such as is presented by invertebrate tissues in general, may well prove to contain potential substrates of arginase, and it is therefore of the greatest importance that any newly discovered bases allied to guanidine should be submitted to its action, if the significance of such results as those of Arnold and Luck is to remain unmodified.

In view of the structural relationship between arcain and arginine, and especially since arcain is attacked by *Aspergillus* arginase, I have submitted arcain to the action of an arginase preparation obtained from fresh guinea-pig liver according to the directions of Edlbacher and Röthler [1925]. The arcain solutions, with added arginase, were incubated overnight at 37° with glycine buffer at $p_H$ 9 under a layer of toluene. Next day, after removal of the proteins, urea was estimated in the filtrates by the manometric technique of Krebs and Henseleit [1932]. The amount of urea found never exceeded that in controls carried out on distilled water, and corresponded to the quantities to be expected from the amounts of arginase taken. Controls performed upon equivalent quantities of arginine showed that this substance was completely hydrolysed by the enzyme preparation in half an hour.

It is therefore concluded that arcain is not attacked by mammalian arginase, and that the results of Arnold and Luck and of the other workers to whom we have alluded must stand, unless subsequent work may show that other substrates of arginase are present in invertebrate materials.

The author's thanks are due to Prof. D. Ackerman, who kindly provided a sample of arcain, and to the Royal Commission for the Exhibition of 1851 during the tenure of whose Senior Studentship these results were obtained.

REFERENCES.

Arnold and Luck (1933). *J. Biol. Chem.* **99**, 677.
Baldwin (1933). *J. Exp. Biol.* **10**, 222.
—— and Needham, D. M. (1933). *J. Physiol.* **80**, 221.
Borsuk, Kreps and Verjbinskaya (1933). *J. Physiol.* U.S.S.R. **16**, 782.
Edlbacher and Röthler (1925). *Z. physiol. Chem.* **148**, 264.
Haldane (1930). Enzymes, p. 107 (Longmans, Green and Co., London).
Kiech, Luck and Smith (1931). *J. Biol. Chem.* **90**, 677.
Kiesel (1921, 1). *Z. physiol. Chem.* **118**, 277.
—— (1921, 2). *Z. physiol. Chem.* **118**, 284.
Krebs and Henseleit (1932). *Z. physiol. Chem.* **210**, 33.
Kutscher and Ackermann (1931). *Z. physiol. Chem.* **203**, 132.
—— —— and Flössner (1931). *Z. physiol. Chem.* **199**, 273.
—— —— and Hoppe-Seyler (1931). *Z. physiol. Chem.* **199**, 277.
Meyerhof (1928). *Arch. Sci. Biol.* **12**, 536.
—— and Lohmann (1928). *Biochem. Z.* **196**, 22.
Needham, D. M., Needham, J., Baldwin and Yudkin (1932). *Proc. Roy. Soc. Lond.* B **110**, 260.
Thomas (1913). *Z. physiol. Chem.* **88**, 465.
—— Kapfhammer and Flaschenträger (1922). *Z. physiol. Chem.* **124**, 75.

# CLVI. THE RECOVERY OF GUANIDINE FROM THE BLOOD-STREAM AFTER INJECTION.

By JOHN ADAMSON SAUNDERS.

*From the Department of Physiology and Biochemistry, University of Durham College of Medicine, Newcastle-on-Tyne.*

(*Received May 30th, 1934.*)

In a previous paper [Saunders, 1932], a method was elaborated for the isolation of guanidine added to shed blood. In the following experiments, the method has been applied to blood from dogs which had been injected with guanidine. It has also been applied to shed blood, with guanidine added to give a concentration equivalent to that produced by the injection. In each case the concentration of guanidine was 90 mg. guanidine per 100 ml. Graphic records of carotid blood pressure were taken throughout each experiment to ascertain whether the action of the drug was typical or not.

## Experimental.

Dogs were anaesthetised with ether 2 hours after the subcutaneous injection of morphine hydrochloride (25 mg. per kg. body weight).

Arterial blood pressure was recorded from the right common carotid artery and injections of guanidine were made through a cannula in the left external jugular vein. Each injection was followed at once by 3 ml. 0·9 % sodium chloride solution, to wash in the guanidine. The dose of guanidine was equivalent to 75 mg. base per kg. The guanidine solutions were made from pure guanidine carbonate (A.R.) neutralised with *N* hydrochloric acid, the excess carbon dioxide being removed by warming.

At a definite interval after the injection, the animals were bled as completely as possible from the common carotid artery, and the blood was mixed with half its volume of 1 % sodium oxalate solution. The average volume of blood obtained in 16 experiments was 38 ml. per kg. Of the 16 animals, 12 gave amounts between 35 and 41 ml. per kg., the remaining 4 giving 19, 33, 47 and 54 ml. per kg.

The details of one typical experiment were as follows:

| | |
|---|---|
| 18. vii. 33. | Dog 4, female, weight 8 kg. |
| 9.0 a.m. | 0·2 g. morphine hydrochloride, subcutaneously. |
| 11.0 a.m. | Ether. |
| 11.30 a.m. | Blood pressure, 91 mm. Hg. Heart rate 196 per minute. |
| 11.31–2 a.m. | 0·9 g. guanidine carbonate, neutralised with HCl in 14 ml., 3 ml. saline. B.P. 66 mm. Hg. Heart rate 204 per min. |
| 11.34 a.m. | B.P. 83 mm. Hg. Heart rate 165 per minute. |
| 11.38 a.m. | Bled from the common carotid artery into 200 ml. 0·6 % sodium oxalate solution. Volume of blood obtained 375 ml. |

Weight picrate isolated 52 mg. After recrystallisation from water, M.P. was 313–315°. (Correction + 8°) = 322°. Microscopically, guanidine picrate.

25·2 mg. on analysis gave basic N 14·4 %, picric acid 80·3 %.

*Results.*

The intravenous injection of 75 mg. guanidine per kg. into a dog would give a concentration of guanidine in the blood of 90 mg. per 100 ml.

Table I shows that when amounts of this order are added to shed blood, the recovery, with the method used, is about 25 %.

Table I.

| Guanidine added to 100 ml. shed blood mg. | Picrate obtained mg. | Guanidine per 100 ml. blood mg. | Recovery % | Analysis | | M.P. ° C. |
|---|---|---|---|---|---|---|
| | | | | Base N % | Picric acid % | |
| 140 | 170 | 35 | 25 | 13·6 | 78·9 | 320 |
| 90 | 148 | 30 | 33 | 12·5 | 78·0 | 313 |
| 46·5 | 54 | 11 | 23 | *15·8 | 77·0 | 306 |

* Analysis and M.P. on crude picrate.

Guanidine was injected into 13 dogs. No guanidine was obtained from the blood of five animals, bled between 15 and 30 minutes after injection. A few crystals (which resembled guanidine picrate microscopically) were obtained from the blood of one dog bled 10 minutes after injection. The yields from animals bled after shorter intervals are given in Table II.

Table II.

| Dog | Volume of blood ml. | Minutes after injection | Picrate obtained mg. | Guanidine per 100 ml. blood mg. | Analysis | | M.P. ° C. |
|---|---|---|---|---|---|---|---|
| | | | | | Base N % | Picric acid % | |
| 4, f. | 375 | 7 | 52 | 2·87 | 14·4 | 80·3 | 322 |
| 7, m. | 510 | 7 | 60 | 2·46 | 13·6 | 79·9 | — |
| *a*, m. | 190 | 5 | 57 | — | * 8·6 | 80·1 | 317 |
| 2, f. | 205 | 5 | 48 | 4·92 | 11·2 | 79·2 | 318 |
| 3, f. | 300 | 5 | 37 | 2·46 | 13·9 | 79·8 | 321 |
| 1, m. | 175 | 3 | 16 | 1·85 | Guanidine picrate microscopically | | — |
| 8, m. | 560 | 3 | 70 | 2·56 | 13·4 | 80·5 | — |

* Analysis on crude picrate, M.P. after recrystallisation.

Guanidine picrate: M.P. 317–323°. Base N 14·8 %, picric acid 79·5 %.

The vascular effects produced by the injection of 75 mg. guanidine per kg. were similar to those found by Alles [1926]. A fall in blood pressure of 20–40 mm. Hg was the immediate effect, the minimum pressure being usually within a minute of the injection. This was followed by a rise in pressure to the pre-injection level, or slightly higher, within 3 or 4 minutes. The heart rate was increased while the blood pressure was low but became slower as the pressure rose.

These results show clearly that guanidine is removed rapidly from the blood-stream after injection, none being recovered after 15 minutes. Between 3 and 7 minutes after injection some guanidine could be isolated, but the average amount was only one-tenth of that recovered when an equivalent amount of guanidine had been added to shed blood.

Of the possible mechanisms for removal of guanidine from the blood, the liver is suggested by the work of Cutler [1931], who has also shown that the accumulation of guanidine in the blood is not due to lack of kidney function. That guanidine is rendered inactive by contact with muscle has been shown by

Camis [1909], and that it is stored in various organs by Ellis [1928]. Paton [1916] suggested that the amount of guanidine in the blood is controlled by the parathyroid glands. It is hoped that experiments now in progress will show which of these methods of removal is the most probable.

SUMMARY.

Guanidine has been isolated from the blood of dogs within 7 minutes after the intravenous injection of 75 mg. guanidine base per kg. body weight. From blood obtained more than 10 minutes after the injection, no guanidine could be recovered.

The expenses of this work have been defrayed by a Medical Research Council grant to Prof. Burns.

I am indebted to Prof. Burns for his continued interest and valuable criticism.

REFERENCES.

Alles (1926). *J. Pharm. Exp. Ther.* **28**, 251.
Camis (1909). *J. Physiol.* **39**, 73.
Cutler (1931). *J. Pharm. Exp. Ther.* **41**, 337.
Ellis (1928). *Biochem. J.* **22**, 930.
Paton (1916). *Quart. J. Exp. Physiol.* **10**, 377.
Saunders (1932). *Biochem. J.* **26**, 801.

# CLVII. THE DETERMINATION OF IRON IN BLOOD-PLASMA.

By FRANK SCOTT FOWWEATHER.

*From the Department of Pathology and Bacteriology, University of Leeds.*

(*Received June 5th, 1934.*)

A NUMBER of investigators have made determinations of iron in blood-serum, chiefly with the object of ascertaining what changes, if any, occur in the non-haemoglobinous iron of the blood in the anaemias. Most of the published methods involve the precipitation of the serum-proteins and the determination of iron in the protein-free filtrate. As a method for determining the iron present in solution in the blood (apart from that present in the cells) the use of protein-free filtrates of serum is open to two objections. Firstly there is the possibility of the removal of some iron in the clot when serum is formed, and secondly iron may be removed in the protein precipitate. Whether such removal does or does not occur, can only be ascertained by experiment, for as yet we have no knowledge of the chemical nature of the iron in question and so cannot infer from the known constitution and properties of the compounds we are concerned with the extent to which these objections are valid. Hence determinations of the iron in plasma are preferable to determinations made on protein-free filtrates of serum, unless it can be demonstrated that the objections just mentioned are in practice of no account; and determinations on plasma are necessary in order to investigate the practical value of these objections.

Some time ago I published a method for the determination of iron in blood, tissues and urine [1926] and made preliminary experiments to see if this method could be modified so as to be applied to blood-plasma. These experiments indicated that such a modification was possible, but the matter was not then pursued further. The interest recently directed to the subject, however, and the advisability of developing this method caused me to take up the matter again.

The first difficulty in any such method is to obtain plasma free from haemoglobin. This difficulty does not arise when protein-free filtrates of serum are used, for the small amounts of haemoglobin, which are practically always present in the serum after blood has been allowed to stand long enough to clot, are removed along with the other proteins. The use of anticoagulants is best avoided since they can only exercise their action if properly mixed with the blood, and such mixing causes rupture of some red cells and consequent contamination of the plasma with haemoglobin. By taking blood with a perfectly dry syringe and needle and transferring at once to a centrifuge-tube lined with paraffin wax, clotting can be delayed long enough to obtain satisfactory separation of the plasma from the cells without the use of any anticoagulant. The test I have used for determining the freedom of the plasma from haemoglobin is the benzidine test, and plasma obtained with proper precautions in the way I have mentioned, generally gives a negative result to this test. As will be seen later, the amount of iron in plasma is in the neighbourhood of $1\gamma$ per ml. or 1 part per million. The benzidine test is capable of detecting blood when present in a

dilution considerably greater than 1 in 1,000,000 and blood contains approximately 47 mg. iron per 100 ml., *i.e.* roughly 1 part in 2000. It will be seen therefore that the amount of haemoglobin which might be present in plasma giving a negative benzidine reaction is much too small to have any effect on the iron content of such plasma; in other words a negative benzidine reaction is a sufficiently good criterion of freedom from haemoglobin. Marlow and Taylor [1934], who also describe a method for determining iron in plasma, use spectroscopic examination for the presence of haemoglobin. It is generally agreed that the benzidine test is more sensitive than spectroscopic examination for this purpose, and the fact that their values are over four times those found by the present method would seem to support this. The cause of these increased values appears to be mechanical rupture of some red cells and the consequent presence of some haemoglobin, since the blood is stirred with a glass rod to bring it into contact with potassium oxalate for the purpose of preventing coagulation.

Plasma obtained in the way I have mentioned is completely oxidised by sulphuric acid and 100 volume hydrogen peroxide and the resultant solution is treated with ammonium thiocyanate. Acetone is added so that the final solution (made up to a fixed volume) contains 50 % by volume of acetone and the resulting pink solution is compared in a colorimeter with that obtained from a standard solution of iron, acidified with sulphuric acid, similarly treated with ammonium thiocyanate and acetone and made up to the same fixed volume.

During the oxidation of the plasma approximately one-third of the sulphuric acid is lost; hence the amount of acid used in the standard is two-thirds of that originally added to the plasma.

Special precautions must be taken to exclude dust from the solutions and apparatus used, and reagents as free as possible from iron must be selected. In any case blank determinations must be made on all reagents and appropriate corrections made in calculating the final result.

Since the standard itself includes reagents which may contain iron, *viz.* ammonium thiocyanate, sulphuric acid and acetone, the iron content of the standard solution may require correction. Actually, using B.D.H. acetone and ammonium thiocyanate, each of A.R. quality, and redistilled iron-free sulphuric acid supplied by the same firm, the amount of iron in the quantities used for this estimation was found to be negligible, but this fact should never be assumed without actual test. The 100 volume hydrogen peroxide, also supplied by B.D.H., does contain appreciable amounts of iron, for which a correction is necessary.

## Experimental.

10–12 ml. of blood are taken by venipuncture in the usual way, using a 20 ml. Record syringe and iridio-platinum needle. The syringe and needle are dried by drawing in and expelling first methylated spirit, then ether and finally air. The needle must be a well-fitting one, since air leaking in at the joint between syringe and needle, and passing as small bubbles through the blood, tends to cause rupture of red cells. The blood must be drawn into the syringe in a slow, steady stream. The needle is then detached and the syringe is held vertically to allow any air in the blood to rise to the surface. The blood is then expelled, again in a slow, steady stream, into an ordinary conical centrifuge-tube, previously lined with paraffin wax, until only 2 ml. remain in the syringe. This quantity, which includes the upper surface of the blood in the syringe, may contain a few bubbles or a little froth and is rejected. The remainder is centrifuged for at least 10 minutes; 4–5 ml. of plasma will then be found to have separated. Of this, about 2·5 ml. are carefully withdrawn with a teat-

pipette, always keeping the tip of the pipette just below the surface of the plasma. On account of the quantity of blood dealt with, it should never be necessary for the tip of the pipette to approach very closely to the boundary between plasma and cells. The plasma drawn off is placed in a test-tube, and 2 ml. are at once measured out and transferred to a pyrex test-tube (200 × 25 mm.). The remainder is submitted to the benzidine test, which, if the above instructions have been carefully followed, will generally be found to give a negative result. If positive, the plasma is of course rejected. To the plasma in the pyrex tube are added 1·5 ml. of sulphuric acid, 0·5 ml. of the 100 volume hydrogen peroxide and a small piece of platinum wire to facilitate smooth boiling. The tube is clamped at an angle of 40° to the horizontal and the contents are heated with a micro-burner until boiling occurs. After a few moments of cautious heating, boiling may be allowed to become vigorous, while water is being driven off, but as soon as charring begins, heating must again be very cautious, as at this stage spitting is liable to occur. During the next few moments charring increases and some carbon is deposited on the sides of the tube. White fumes then begin to be evolved and become copious and the liquid begins to froth but not dangerously. In fact, at this stage, heating may again become vigorous as any danger from spitting is now over. After this stage the white fumes get much less and sulphuric acid is seen to condense just above the liquid and run back, removing much of the carbon deposited on the tube. The liquid is now allowed to cool for about 1 minute. 0·5 ml. of the hydrogen peroxide is now added, drop by drop, and the liquid again heated. A brisk evolution of oxygen occurs. Heating is continued until sulphuric acid is again seen to be condensing on the walls of the tube and running back. At this stage all carbon deposit should be removed, and the liquid be transparent and reddish brown in colour. After cooling again for a short time, 0·5 ml. of hydrogen peroxide is again added and heating resumed. The liquid now becomes pale yellow. On the further addition of 0·5 ml. hydrogen peroxide and heating again, the liquid becomes entirely colourless. Heating is continued for a few moments after the loss of all colour, to decompose the hydrogen peroxide thoroughly. The liquid is then allowed to cool completely, after which it is diluted with about 2 ml. of water and washed into a 20 ml. stoppered graduated flask. The total volume of the liquid and washings must not exceed 8 ml. Meanwhile, in a similar flask are placed 1 ml. of sulphuric acid, 0·5 ml. of a standard iron solution containing 0·01 mg. of iron per ml. and 5–6 ml. of water. To both flasks are added 10 ml. of acetone and the contents thoroughly mixed. Some rise of temperature occurs, and the flasks are set aside until the contents have cooled to room temperature. To each are then added 2 ml. of 3*M* ammonium thiocyanate solution and the contents mixed and made up to the mark. The two solutions are then compared in a colorimeter.

I have found illumination of the colorimeter by artificial light unsatisfactory for this comparison and have therefore adhered to daylight illumination throughout.

*Preparation of standard iron solution.*

A stock solution containing 0·1 mg. of iron per ml. is first prepared. 0·7 g. pure ferrous ammonium sulphate is dissolved in about 50 ml. of water. 20 ml. of 10 % iron-free sulphuric acid are added, the solution is slightly warmed and 0·1*N* potassium permanganate solution added to oxidise the ferrous salt completely. The solution is then diluted to 1 l.

The required standard is prepared from this solution by making a 1 in 10 dilution with water.

*Determination of blanks.*

(*a*) *For standard solutions.* Two different quantities of the standard iron solution, *e.g.* 0·5 ml. and 0·7 ml., are each treated with sulphuric acid, acetone and ammonium thiocyanate as above described and compared in the colorimeter.

If $A$ and $B$ are the amounts of iron present in the standard solution taken, $a$ and $b$ the colorimeter readings of the solutions prepared from them, and $x$ the amount of iron in the other reagents used, then

$$\frac{A+x}{B+x}=\frac{b}{a}$$

from which $x$ is calculated.

(*b*) *For unknown solutions.* 1 ml. of the standard iron solution is transferred to a pyrex tube and treated by the above method exactly as if it were plasma. It is then compared with a standard made up from the same volume of iron solution, and the difference between the iron content of the two solutions is calculated.

The accuracy of the method was tested by adding to various quantities of serum whose iron content had previously been determined different quantities of the standard iron solution and then determining the total amount of iron present in the mixtures so obtained.

The following results were obtained:

| Iron in serum mg. | Iron added mg. | Total amount present mg. | Total amount recovered mg. |
|---|---|---|---|
| 0·00265 (2 ml.) | 0·0010 (0·1 ml.) | 0·00365 | 0·00353 |
| 0·00199 (1·5 ml.) | 0·0025 (0·25 ml.) | 0·00449 | 0·00444 |
| 0·00211 (1 ml.) | 0·0050 (0·5 ml.) | 0·00711 | 0·00687 |
| 0·00076 (0·5 ml.) | 0·0100 (1 ml.) | 0·01076 | 0·01050 |

The iron content of serum, plasma and protein-free filtrates of serum (prepared by adding an equal volume of 20 % trichloroacetic acid to the serum) were determined by this method in the case of three healthy individuals, with the following results:

| Plasma ($\gamma$ iron per ml.) | Serum ($\gamma$ iron per ml.) | Protein-free filtrate of serum ($\gamma$ iron per ml. serum) |
|---|---|---|
| 1·8 | 2·02 | 1·36 |
| 1·02 | 1·60 | 0·68 |
| 1·14 | 1·58 | 0·72 |

The higher values found for serum are explained by the fact that in all cases the serum was benzidine-positive while the plasma was benzidine-negative. Hence it is not possible to say if any iron is removed by the clot. The lower values for protein-free filtrates of serum than for plasma indicate removal of an appreciable proportion of iron either in the clot or the protein precipitate or both. It is possible that these differences may be greater in certain anaemic conditions than in health.

*Values for iron in plasma by the present method.*

The plasma of 10 male students and 10 female students, all presumably healthy, has been examined by this method. In all cases blood was taken between 10 a.m. and 10.30 a.m., breakfast having been allowed. The iron found in the males varied from 0·95$\gamma$ per ml. to 1·80$\gamma$, the mean being 1·25$\gamma$. In the females the values were found to be between 0·60$\gamma$ and 1·56$\gamma$, the mean being 1·05$\gamma$.

Locke *et al.* [1932], using protein-free filtrates of serum, found an average of 1·00$\gamma$ per ml. of serum for males, and 0·77$\gamma$ for females. In this case blood was taken before breakfast after a 12 hours' fast. Allowing for the difference which I have found between plasma and protein-free filtrates of serum, these figures agree well with those I have just given.

Warburg and Krebs [1927], using a totally different method, report a few values which are in the neighbourhood of 0·7$\gamma$ per ml. of serum. Many of the reported values however are considerably higher. Thus Marlow and Taylor, whose method has already been referred to, found values varying between 4 and 7$\gamma$ per ml. of plasma; and Riecker [1930] found an average of 11$\gamma$ per ml. of serum.

SUMMARY.

A method is described for the determination of iron in blood-plasma. The precautions necessary for obtaining plasma free from haemoglobin are indicated, together with the test which is used to determine if the required freedom from haemoglobin has been obtained. It is shown that the method is capable of a very satisfactory degree of accuracy.

Results are given which show that the iron in plasma is higher than that found in serum when protein-free filtrates of serum are used for analysis. The difference appears to be due to removal of iron in the blood-clot or the protein precipitate.

The results of iron determinations in plasma in the case of 10 males and 10 females are given.

REFERENCES.

Fowweather (1926). *Biochem. J.* **20**, 93.
Locke, Main and Rosbash (1932). *J. Clin. Invest.* **11**, 527.
Marlow and Taylor (1934). *Arch. Int. Med.* **53**, 551.
Riecker (1930). *Arch. Int. Med.* **46**, 458.
Warburg and Krebs (1927). *Biochem. Z.* **190**, 143.

# CLVIII. THE CATAPHORESIS OF MAMMALIAN ERYTHROCYTES.

By FREDERICK OLIVER HOWITT.

*From University College, Nottingham.*

(*Received June 9th, 1934.*)

THE surface charge of the corpuscular elements of the blood is determined by the nature of the corpuscle membrane. It is therefore obvious that investigation of this surface charge is of great service in the elucidation of the constitution and behaviour of the cell surface. Many workers have attacked this problem but in the majority of cases the results have been only of a semi-quantitative nature or have been obtained under ill-defined conditions. Thus Kozawa [1914] recorded that the erythrocytes of various animals could be brought to an isoelectric condition at a reaction characteristic for each animal, the $p_H$ decreasing in the series: rabbit > guinea-pig > cat, man > dog > pig. Kosaka and Seki [1921] found that when suspended in physiological saline the following series in order of increasing velocity of electrophoretic migration was observed: rabbit < pig < guinea-pig < man < mouse < rat, cat, dog. This order was reversed on suspension in 9·5 % sucrose.

That the cataphoretic velocity of the erythrocytes was dependent on the reaction of the surrounding medium was first postulated by Kozawa [1914] and confirmed by Eggerth [1924] and by Netter [1925]. These workers observed that degrees of acidity or of salt concentration which were comparatively far removed from the physiological range resulted in a changing velocity indicating an unstable condition due to damage of the cell membrane.

Bernardi [1926] recorded the following series for the rates of cataphoretic migration: rabbit < cat < guinea-pig < man < pig, a series which was not in entire agreement with that of Kosaka and Seki. The whole question has been recently placed on a sound basis by the excellent work of Freundlich and Abramson [1927]. These workers established the fact that the velocity of erythrocytes and leucocytes of different adult horses when suspended in serum or plasma did not vary greatly, although the velocity of the red cell was markedly different from that of the white cell.

Abramson [1929] has carried out an extensive investigation of the cataphoretic velocity of erythrocytes and this investigation will be referred to in detail owing to its importance as the first research of this type performed under standard conditions and also to the fact that the experiments about to be described were based on his technique.

Abramson made use of the micro-cell of Northrop and Kunitz [1925]. One drop of the freshly drawn blood was added to 25–50 ml. of $M/15$ phosphate buffer at $p_H$ $7{\cdot}35 \pm 0{\cdot}03$. Measurements were made at room temperatures (22–27°) and corrected to 25° on the assumption that the temperature coefficient of the cataphoresis of the red cell in the medium employed was 0·02 per ° C. No significant change over a period of 24 hours occurred in the velocity of the red cells investigated when suspended in the above phosphate buffer. The

following values were obtained for the cataphoretic velocities of mammalian erythrocytes, expressed as $\mu$/sec., volt/cm. and corrected to 25°. In every instance the cell was negatively charged and hence the movement was towards the anode: rabbit, 0·55; sloth, 0·97; pig, 0·98; opossum, 1·07; guinea-pig, 1·11; man, 1·31; monkey, 1·33; mouse, 1·40; cat, 1·39; rat, 1·45; dog, 1·65. The order, but not the absolute values, was the same in buffered isotonic glucose. No relationship between zoological species and cataphoretic velocity was demonstrable. With regard to human erythrocytes no marked difference in velocity could be detected following the incidence of pregnancy or of severe anaemia, although in the latter instance distinct changes in the morphology of the cells were present.

Perhaps the most important observation during this work was that the red cells, unlike the leucocytes, appeared to be incapable of any surface union with the proteins of the corresponding plasma. Thus human and rabbit erythrocytes exhibited the same difference in velocity when compared in rabbit or human serum. Again, whilst cholesterol crystals and quartz particles adsorbed gelatin from dilute solution in the phosphate buffer, the erythrocytes, even after 24 hours' contact with the gelatin solution, retained their original velocity.

In view of the importance of Abramson's results it was decided to endeavour to determine the velocities of various mammalian erythrocytes with the aid of the macro-apparatus described elsewhere [Prideaux and Howitt, 1930].

## Experimental.

A stock solution of $M/15$ phosphate buffer was prepared from A.R. $KH_2PO_4$ and $Na_2HPO_4$. The reaction was determined by means of the hydrogen electrode and adjusted to $p_H$ $7{\cdot}37 \pm 0{\cdot}02$. The apparatus was used with secondary electrodes as described in the experiments on insulin and serum-globulin [Howitt and Prideaux, 1932, 1, 2]. The lower part of the U-tube was filled with a suspension of whole blood in the buffer (approximately 0·5 ml. of blood added to 30 ml. of buffer) whilst the upper part was filled in the usual way with buffer alone. Following levelling and opening of the main taps, the tap at the bottom of the U-tube was carefully opened so that the two surfaces of separation slowly sank until they reached levels approximately agreeing with the lower 10 mm. graduation of each limb, at which point the lower tap was closed. With care this adjustment of the levels could be performed with an excellent retention of the surface boundaries. The erythrocytes were thus acting as self-indicators of their cataphoretic velocities. The cataphoresis was then carried out at room temperature, an E.M.F. of about 100 volts being applied at the secondary electrodes in order to balance the potential due to the mains applied at the primary electrodes. The samples of blood were collected not longer than 12 hours before the actual experiment.

In calculating the actual velocity from the "up" and "down" readings consideration must be given to the effect of gravity, *i.e.* to the velocity of sedimentation of the erythrocytes. It may be noted that this correction was not needed in the micro-method used by Abramson and Freundlich, since in these experiments the particles were viewed from above and only the horizontal component of velocity, *i.e.* that due to the electric field, was actually measured.

In the case of the macro-apparatus described above this sedimentation velocity must be taken into consideration and this is achieved by a simple calculation on the "up" and "down" velocities. Thus, supposing the true *mean* velocity of the corpuscle is $x$ and the rate of sedimentation is $y$, the "up"

velocity would actually be observed as $x-y$ and the "down" velocity as $x+y$. From this it follows that the true mean velocity is given by half the algebraic sum of the observed "up" and "down" velocities. Thus as an example:

(*a*) Velocity in + electrode limb = 6 mm./hr. down.
(*b*) Velocity in − electrode limb = 22 mm./hr. down.

The value for (*a*) must obviously be expressed as $-6$ for in this case $y$ is greater than $x$. Hence the true mean velocity is $(22-6)/2=8$ mm./hr.

Incidentally the sedimentation velocity in this particular example is 14 mm./hr. or nearly twice that of the cataphoretic migration.

In converting the true mean velocity into a value expressed in $\mu$/sec., volt/cm. it was considered justifiable on the ground of the approximate equality of conductivity of suspension and plain buffer to modify the Kruyt equation (i) to the simplified form (ii)

$$\text{(i)}\quad v=\frac{w_1h_1+w_2h_2}{tE};$$

$$\text{(ii)}\quad v=\frac{v'(h_1+h_2)}{tE};$$

where $v'$ is the true mean velocity.

Now $h_1+h_2=274$ and hence the equation becomes

$$v=\frac{274\times v'}{tE},$$

where $t$ and $E$ have their usual significance.

Thus, completing the example given above,

$$v'=8 \text{ mm.}\quad t=3600 \text{ secs.}\quad E=110 \text{ volts.}$$

$$v=\frac{274\times 0{\cdot}8}{3600\times 110}\times 1000=0{\cdot}53\,\mu\text{/sec., volt/cm.}$$

The cataphoretic velocities of the erythrocytes of the following animals were determined: rat, rabbit, mouse, guinea-pig.

In the case of mouse-blood a determination was made on a suspension in the presence of insulin (0·02 %) and also on a suspension of blood from mice suffering from hypoglycaemic convulsions induced by administration of insulin. With each of these two determinations three experiments were carried out (at 37°) using suspensions of blood alone and of blood in the presence of glucose (0·1 %) and of glucose (0·1 %) together with insulin (0·02 %) respectively.

A further determination was made on blood from mice which had been used for the testing of complex arsenic derivatives and which, in the normal course of events, would have died within a few days from arsenical poisoning.

These results, expressed in $\mu$/sec., volt/cm., are given in Table I.

On the whole the agreement between the results yielded by the micro- and macro-methods is excellent. An obvious advantage of the macro-method is that the observation of a large number of cells is achieved and hence the average behaviour of a single cell is obtained with a greater accuracy than is derived from the observation of a solitary corpuscle. There appears to be a tendency for the macro-method to yield slightly higher values.

It will be observed that the value given by Abramson for the mouse (1·35 at 25°) is equivalent to one of 1·74 when corrected to 37° whilst the corresponding values obtained by the macro-method are 1·42 and 1·86 respectively. Actual determination at 37° gives a value of approximately 1·9 and hence it appears probable that the value of 1·42 (at 25°) is more acceptable than that of 1·35. In addition the fact that a temperature coefficient of approximately

Table I. *Cataphoretic velocities of some mammalian erythrocytes in M/15 phosphate buffer at* $p_H$ *7·37 ± 0·02.*

I. *Rat.* (Abramson—1·45 at 25°.)

| Animal No. | $v$ | Temp. | $v$ (25°) |
|---|---|---|---|
| 1 | 1·15 | 16 | 1·43 |
| 2 | 1·34 | 17·5 | 1·59 |
| 3 | 1·02 | 16 | 1·28 |
| 4 | 1·22 | 14·5 | 1·56 |
| 5 | 1·09 | 14·5 | 1·41 |
| | | Average | 1·45 |

II. *Rabbit.* (Abramson—0·55 at 25°.)

| Animal No. | $v$ | Temp. | $v$ (25°) |
|---|---|---|---|
| 1 | 0·53 | 15 | 0·68 |
| 2 | 0·52 | 18 | 0·61 |
| | 0·42 | 18 | 0·49 |
| 3 | 0·49 | 15 | 0·62 |
| | 0·49 | 15 | 0·62 |
| | | Average | 0·60 |

III. *Mouse.* (Abramson—1·35 at 25°.)

| Animal No. | $v$ | Temp. | $v$ (25°) | $v$ (37°) |
|---|---|---|---|---|
| 1 | 1·16 | 15 | 1·44 | 1·89 |
| 2 | 1·12 | 15 | 1·40 | 1·82 |
| 3 | 1·94 | 37 | — | 1·94 |
| | | Average | 1·42 | |

"Arsenical mouse"

| Animal No. | $v$ | Temp. | $v$ (25°) | $v$ (37°) |
|---|---|---|---|---|
| 1 | 1·16 | 15 | 1·44 | 1·89 |
| 2 | 1·16 | 15 | 1·44 | 1·89 |
| | 1·14 | 16 | 1·42 | 1·86 |
| | | Average | 1·43 | |

Normal mouse (insulin-glucose experiment). Determinations at 37°.

| | |
|---|---|
| Blood alone ... ... ... ... ... | 1·84 |
| Blood—glucose ... ... ... ... ... | 1·92 |
| Blood—glucose—insulin ... ... ... | 1·95 |

Insulinised mouse (insulin-glucose experiment). Determinations at 37°.

| | |
|---|---|
| Blood alone ... ... ... ... ... | 1·92 |
| Blood—glucose ... ... ... ... ... | 1·98 |
| Blood—glucose—insulin ... ... ... | 1·77 |

IV. *Guinea-pig.* (Abramson—1·11 at 25°.)

| Animal No. | $v$ | Temp. | $v$ (25°) |
|---|---|---|---|
| 1 | 0·98 | 18 | 1·16 |

Summarising the average values we obtain the following results (corrected to 25°) for normal animals, the corresponding values of Abramson being given in parenthesis for comparison. These figures indicate the velocity of the corpuscles towards the anode.

| | |
|---|---|
| Rat ... ... ... ... ... ... | 1·45 (1·45) |
| Rabbit ... ... ... ... ... | 0·60 (0·55) |
| Mouse ... ... ... ... ... | 1·42 (1·35) |
| Guinea-pig ... ... ... ... ... | 1·16 (1·11) |

2 % is applicable to the cataphoretic velocity of red blood corpuscles indicates that the surface potential of the corpuscle does not depend directly on the vitality of the cell inasmuch as the processes of the living cell are generally associated with a greater and less regular temperature coefficient. In this con-

nection an investigation of the temperature coefficient of cataphoretic velocity of the erythrocytes of some poikilotherms (*e.g.* the frog) would be of considerable importance, for comparison with the above homoeotherms could then be made.

An observation of some interest was made in a few experiments where the erythrocytes had partly haemolysed. In these experiments the intact cells migrated independently of the oxyhaemoglobin. The final result was the formation of three well-defined layers (which could be seen most clearly when a bright but diffused light was placed behind the apparatus) consisting of the colourless buffer, the transparent red solution of oxyhaemoglobin and the opaque suspension of intact cells. The oxyhaemoglobin showed no marked tendency to migrate: a slight wandering occurred sometimes to the cathode and sometimes to the anode. It should be noted that Geiger [1931] has claimed the isolation by cataphoresis of two different oxyhaemoglobins from the blood of some animals.

The opinion has been expressed that the surface potential of the erythrocyte appears to be a function solely of the constituents of the surface membrane and not to be correlated with the vital processes occurring at the interface. This is substantiated by the normal values obtained for the velocities of red corpuscles of the blood of mice suffering from arsenical poisoning. Arsenic-containing substances are well known to inhibit the oxygen uptake of certain tissues, and Onaka [1911] has recorded that arsenite, like cyanide, strongly inhibits the oxygen uptake of avian erythrocytes. Unfortunately facilities were not at hand to determine whether the oxygen uptake of the corpuscles actually used was below normal.

The cataphoretic velocity of mouse erythrocytes is but little changed by the presence of 0·1 % glucose or of 0·1 % glucose together with 0·02 % insulin. Abramson recorded an increase from 1·35 (in plain phosphate buffer at $p_H$ 7·38) to 2·60 (in isotonic glucose and buffer at $p_H$ 6·80). With mice suffering from hypoglycaemic convulsions there occurs a decrease in the velocity due to the presence of both glucose and insulin. This finding is of considerable importance and will again be referred to in the consideration of the rôle of the erythrocytes in the action of insulin.

From the above it is clear that the use of a macro-apparatus gives fairly constant and accurate results for the cataphoretic velocities of mammalian erythrocytes. This view leads to a fair degree of confidence in the undertaking of an investigation of the cataphoresis of human erythrocytes and their part in the action of insulin in the organism, an investigation which will be described in a further communication.

SUMMARY.

Values for the cataphoretic migration of mammalian erythrocytes (rat, rabbit, mouse, guinea-pig) at 22–27° in $M/15$ phosphate buffer were determined by means of a macro-apparatus and the results compared with those obtained by Abramson using a micro-method.

It affords the author pleasure to record his gratitude to Dr E. B. R. Prideaux for his advice and guidance during the course of the above work.

## REFERENCES.

Abramson (1929). *J. Gen. Physiol.* **12**, 711.
Bernardi (1926). *Arch. Sci. Biol.* **8**, 1.
Eggerth (1924). *J. Gen. Physiol.* **6**, 587.
Freundlich and Abramson (1927). *Z. physikal. Chem.* **128**, 26.
Geiger (1931). *Proc. Roy. Soc. Lond.* B **107**, 368.
Howitt and Prideaux (1932, 1). *Proc. Roy. Soc. Lond.* B **112**, 13.
—— —— (1932, 2). *Proc. Roy. Soc. Lond.* B **112**, 24.
Kosaka and Seki (1921). *Comm. Okayama Med. Soc.* No. 372.
Kozawa (1914). *Biochem. Z.* **60**, 146.
Netter (1925). *Pflüger's Arch.* **208**, 16.
Northrop and Kunitz (1925). *J. Gen. Physiol.* **7**, 729.
Onaka (1911). *Z. physiol. Chem.* **70**, 433.
Prideaux and Howitt (1930). *J. Sci. Instr.* **7**, 90.

# CLIX. THE QUANTITATIVE DETERMINATION OF INDOLE IN BACTERIAL CULTURES.

By FRANK CHARLES HAPPOLD and LESLIE HOYLE.

*From the Department of Pathology and Bacteriology, University of Leeds.*

(*Received June 11th, 1934.*)

Methods of estimating indole in bacterial cultures have been devised by various workers, notably by Herter and Foster [1905–06] and Fellers and Clough [1925].

In these methods the indole is separated from the culture by steam-distillation, the distillate being treated with Ehrlich's *p*-dimethylaminobenzaldehyde reagent and the indole estimated colorimetrically by comparison with the colour given by standard indole solutions. It is important not to use excess of Ehrlich's reagent since this causes a change in the colour. The methods are somewhat laborious and not very accurate, as even under optimum conditions the whole of the indole present is not recoverable in the distillate, and with unknown concentrations it is often difficult to determine the correct amount of Ehrlich's reagent to use.

In the course of some investigations carried out by one of us (L. H.) it was found necessary to estimate indole present in various complex bacterial cultures, notably in cultures containing bile and bile salts, which could not be distilled on account of excessive frothing, and a study was therefore made of the possibility of estimating the indole by extraction with organic solvents.

The proposed method was to extract the indole from the culture with the solvent, and estimate the indole present in the extract by the Ehrlich reaction.

The various indole solvents were found to fall into three groups.

(1) *Solvents miscible with Ehrlich's reagent:* ether, chloroform, amyl alcohol, *iso*amyl alcohol, capryl alcohol.

(2) *Solvents immiscible with Ehrlich's reagent but dissolving the rosindole body:* benzene (dissolves the rosindole slowly).

(3) *Solvents immiscible with Ehrlich's reagent and in which the rosindole body is insoluble:* light petroleum, xylene.

Solvents of group (1) were found unsuitable, as it was difficult to decide the amount of Ehrlich's reagent required, and excess was found to alter the colour of the rosindole body.

Solvents of group (2) were imperfect because of the unequal distribution of the rosindole body between the solvent and the Ehrlich's reagent.

Solvents of group (3) were found suitable, and light petroleum, which gave the best results, was easier to handle than xylene.

It was found that extraction of the culture with an equal volume of light petroleum removed about 90 % of the indole present, so that two successive extractions removed practically the whole of the indole. The extraction was equally effective whether the culture were made acid or alkaline, and it was found useful to acidify the cultures, as this facilitated separation of the liquids.

*Technique recommended for estimation of indole.*

10 ml. of culture are acidified with one drop of concentrated HCl and extracted with two successive 10 ml. quantities of light petroleum (B.P. 40–60°). A third extraction can be made to ensure complete removal of indole. The pooled extracts are washed once with 10 ml. of distilled water. The light petroleum is then extracted with successive 5 ml. quantities of freshly prepared Ehrlich's reagent until no more rosindole is produced. The rosindole is completely insoluble in light petroleum and therefore collects exclusively in the layer of Ehrlich's reagent.

The addition of the reagent in 5 ml. quantities at a time ensures that the correct amount is used, a point of considerable importance. If the amount of indole present is very small, of the order of 1 : 500,000, the reagent should be added 1 ml. at a time.

A standard solution of indole is prepared in the same medium as the culture, a concentration of 1 : 50,000 being suitable, and extracted with light petroleum in the same manner. The rosindole from the culture is then compared with that from the standard in Nessler tubes. It is usually necessary to immerse the solutions in boiling water for a few minutes to remove milkiness; this does not affect the result provided both solutions are immersed for the same time.

The method has the advantage of simplicity, it can be used with small quantities of culture, and no difficulty has been experienced with substances interfering with the Ehrlich reaction, as such substances are apparently not extracted by light petroleum. The method is accurate with concentrations of indole down to 1 : 1,000,000.

Comparison of the method with estimations made by the steam-distillation technique has shown the extraction method to be never less accurate if parallel controls are used in each method, *i.e.* if standard solutions are distilled and extracted respectively to compare with the unknowns. If controls are not done, and the distillate and extract are compared directly with standard indole solutions, the extraction method is definitely the more accurate since a greater proportion of the indole present is recoverable by extraction than by steam-distillation.

*Qualitative detection of indole in bacterial cultures.*

The following simple application of the extraction technique has been found useful for detection of indole in cultures. 5 ml. of culture are shaken vigorously with 2 ml. of xylene, and 1 ml. of Ehrlich's reagent is run on to the surface of the mixture, the liquids being allowed to redistribute themselves by gravity. The xylene extracts the indole and as the globules of xylene pass through the layer of Ehrlich's reagent on their way to the surface the rosindole body is formed, and a red ring appears at the lower surface of the xylene layer. Xylene is used in the test in preference to light petroleum, as Ehrlich's reagent mixes uniformly with light petroleum-culture mixture.

The test is between 20 and 50 times as sensitive as the usual method of mixing the culture and the Ehrlich's reagent (with or without the addition of persulphate) and extracting the rosindole with chloroform.

Many bacteriologists detect indole in cultures by the simple method of running a layer of Ehrlich's reagent on to the surface of the culture, when a red ring appears at the junction of the liquids. This test is exceedingly delicate, and apparently owes its great sensitivity to the fact that indole, being a substance which lowers surface tension, becomes concentrated in the surface layer. In the xylene extraction test described above the intensity of the reaction depends

on the concentration of indole in the whole culture, whereas in the ring test the intensity depends on the concentration in the surface layer. The xylene test gives more intense reactions than the ring test with concentrations of indole such as are usually present in bacterial cultures (*e.g.* 1 : 100,000) and is probably the preferable test for such cultures, but the ring test is more delicate, indole being detectable in concentrations with which the xylene test gives negative results. It was found impossible to determine accurately the end-point of the ring test, since when dilutions of indole of the order of 1 : 10,000,000 are reached the concentration of indole in the surface layer becomes an appreciable fraction of the whole amount, and further dilutions cannot be made with accuracy.

The authors are indebted to the Medical Research Council for personal grants and for grants in aid. They take this opportunity of expressing their thanks for this assistance.

REFERENCES.

Fellers and Clough (1925). *J. Bact.* **10**, 105.
Herter and Foster (1905–06). *J. Biol. Chem.* **2**, 257.

# CLX. THE NON-ESSENTIAL NATURE OF TYROSINE.

By ROBERT SAXELBY ALCOCK[1].

*From the Biochemical Department, Cambridge.*

(*Received June 13th, 1934.*)

THE text-book statement that tyrosine is an indispensable amino-acid rests upon the observations of Abderhalden [1913] who showed that a dog, while unable to maintain weight on a diet from which the tyrosine had been almost completely removed, was able to do so when a supplement of tyrosine was given. The same author [1915] extended the work to rats and came to the conclusion that tyrosine is essential to growth and maintenance.

The work on the dog has never been repeated, but both Totani [1916] and Lightbody and Kenyon [1928] have failed to substantiate the findings in the second paper. The former used as nitrogen source acid-hydrolysed caseinogen, freed from tyrosine by crystallisation and supplemented with tryptophan. He found that rats grew well on such a diet and that tyrosine supplements had no effect upon the growth rate. Totani also came to the conclusion, which has not been supported by other workers [see Jackson *et al.*, 1928], that hydrolysed gelatin, with a supplement of tryptophan alone, will enable the rat to maintain itself and even gain in weight. The addition of tyrosine to such a diet had no effect, but the results are too erratic to permit of any conclusions being drawn.

Lightbody and Kenyon used a mixture of hydrolysed hair and gelatin—the choice of proteins being governed by a desire to keep the phenylalanine content low. Their rats grew—not normally—but the growth was in no way affected by tyrosine supplements. They came to the conclusion that tyrosine is not an essential amino-acid.

There are several difficulties in this problem. The first is that of removing tyrosine quantitatively from a hydrolysate; the second is the impossibility of removing phenylalanine; and a third is the unsatisfactory nature, both of gelatin, and of hydrolysed proteins, however supplemented, from a nutritional point of view. In the present work the first two of these difficulties have been overcome, and the failure to settle the question finally is due to the last.

A tyrosine-free amino-acid mixture was prepared as follows: 800 g. of caseinogen were digested for 3 weeks with pig's pancreas. The digest was then adjusted to $p_H$ 4·6 with hydrochloric acid and allowed to stand in the ice-chest for 2 days. The tyrosine was filtered off (26 g. crude product), the filtrate concentrated *in vacuo* to 4 litres and the tyrosine again crystallised out. The filtrate from this was diluted to 10 litres, adjusted to $p_H$ 7·6 and treated, in two lots of 5 litres, with 2 g. of catechol and the juice from 1·5 kg. of potatoes, added in 50 ml. doses at intervals, for some 18 hours. The fluid was kept well stirred, and air was passed in continuously. Further 2 g. of catechol were added during the time. The Millon reaction was followed and also that obtained by adding

[1] Senior 1851 Student.

fresh tyrosinase and a trace of catechol to a sample. The former reaction never entirely disappeared—it only became weak and atypical, but the latter at the end of the 18 hours had entirely gone.

The two lots were reunited and boiled with charcoal, and after filtration evaporated to dryness, the first stages in an open pan, and the last in a vacuum-oven. The product was ground to a fine powder, and made up into a diet of the following composition:

| | |
|---|---|
| Amino-acid mixture ... ... | 18 % |
| Starch ... ... ... ... | 42 % |
| Sugar ... ... ... ... | 20 % |
| Fat (palm kernel oil) ... ... | 15 % |
| Salt mixture ... ... ... | 5 % |

Vitamins were given in the form of 3 drops of cod-liver oil and an alcoholic extract of autolysed yeast, equivalent to 0·75 g. of fresh yeast per rat daily. The latter contains vanishingly small quantities of tyrosine.

Two groups of young black-and-white rats were used four in each group. The diet was fed *ad lib.*, and to the second group 100 mg. tyrosine were given between the four animals daily. The amount used corresponds with the minimum tryptophan requirement of 20 mg. daily. There were two of each sex in each group. The experiment lasted 35 days.

Table I.

| | Rat | Sex | Initial weight g. | Final weight g. | Gain g. | Average |
|---|---|---|---|---|---|---|
| Group I | 1 | ♂ | 69 | 110 | 41 | |
| | 2 | ♀ | 72 | 122 | 50 | |
| | 3 | ♂ | 80 | 119 | 39 | |
| | 4 | ♀ | 75 | 119 | 44 | 43·5 |
| Group II | 5 | ♀ | 73 | 111 | 38 | |
| | 6 | ♀ | 84 | 130 | 46 | |
| | 7 | ♂ | 62 | 110 | 48 | |
| | 8 | ♂ | 71 | 109 | 38 | 42·0 |

Rats 5, 6, 7 and 8 received tyrosine.

It will be seen that there is no difference between the growth rates of the two groups.

The experiment was repeated, using caseinogen which had been hydrolysed by means of 25 % sulphuric acid in an autoclave for 3 hours. The hydrolysate, after removal of sulphuric acid with baryta, was treated as above to free it from tyrosine. In this case, the fluid was boiled with charcoal just before treatment with the tyrosinase, and the reaction required only 12 hours. The repetition was made necessary by the possibility of small quantities of tyrosine having been introduced with the pancreas, and of undigested tyrosine peptides, which the tyrosinase will not attack.

The experiment was carried out as before, but the results were slightly less regular (Table II). In addition to the vitamins, a supplement of 20 mg. of tryptophan per rat was given daily, to compensate for that destroyed in the acid hydrolysis. The experiment was carried on for 30 days.

It thus appears that the addition of tyrosine to diets deficient therein is without effect on the growth rate of rats, and tyrosine must accordingly be classed as a non-essential amino-acid. But Embden and Baldes [1913] showed that in the surviving liver of the dog tyrosine is probably formed from phenylalanine, and there is no doubt that the two amino-acids may follow similar lines

Table II.

| | Rat | Sex | Initial weight g. | Final weight g. | Gain g. | Average |
|---|---|---|---|---|---|---|
| Group I | 1 | ♂♀♂♀ | 82 | 120 | 38 | |
| | 2 | | 80 | 108 | 28 | (of 3) |
| | 3 | | 78 | 111 | 33 | 30·3 |
| | 4 | | 66 | 75 | 9 | |
| Group II | 5 | ♀♂♀♂ | 75 | 108 | 33 | |
| | 6 | | 70 | 107 | 37 | |
| | 7 | | 70 | 100 | 30 | |
| | 8 | | 68 | 91 | 23 | 30·1 |

Rats 5, 6, 7 and 8 received tyrosine.
Rat 4 was abnormal, and has been ignored in the calculation of the average gain in weight.

of metabolism. They ought therefore to be treated as a group, with regard to indispensability, and indeed phenylalanine may well be the more important, for while it is possible for tyrosine to be produced from phenylalanine, the reverse change is less probable.

There is no method for removing phenylalanine from amino-acid mixtures, as there is for tyrosine, or for destroying it, as for tryptophan. A phenylalanine-free diet requires a phenylalanine-free protein, and the only one described in the literature is the keratin of goose-feathers [Abderhalden and le Conte, 1905]. It was decided therefore to attempt to feed rats on a hydrolysate of goose-feathers, and to test the effect of the addition of tyrosine and of phenylalanine to such a diet.

In order to render the keratin digestible, as well as for the removal of the tyrosine, it is necessary to hydrolyse it, and this is not possible with enzymes. Three different hydrolysates were prepared. For the first, 500 g. of goose-feathers were boiled for 18 hours with 2 litres of 25 % sulphuric acid, and after neutralisation with baryta and filtration, the mixture was freed from tyrosine as above. The resulting powder was an evil-smelling, black, deliquescent substance, and when made up into a diet, proved so unpalatable that the rats would not touch it. A further 500 g. were hydrolysed, this time for 3 hours in an autoclave, also with 25 % sulphuric acid. The material was slightly less repulsive, but even so, the animals would not consume sufficient to maintain balance.

The diet was made up as with the caseinogen, and a supplement of tryptophan was given. Two groups of four were used as before, Nos. 5, 6, 7 and 8 being given tyrosine, and 6 and 7 a similar quantity of phenylalanine in addition for a part of the experiment. Duration of experiment: 30 days.

Table III.

| | Rat | Sex | Initial weight g. | Final weight g. | Loss g. | Average |
|---|---|---|---|---|---|---|
| Group I | 1 | ♂♀♂♀ | 108 | 88 | 20 | |
| | 2 | | 109 | 92 | 17 | |
| | 3 | | 80 | 62 | 18 | |
| | 4 | | 82 | 71 | 11 | 16·5 |
| Group II | 5 | ♀♂♀♂ | 111 | 87 | 24 | |
| | 6 | | 100 | 92 | 8 | |
| | 7 | | 71 | 56 | 15 | |
| | 8 | | 82 | 66 | 16 | 15·75 |

These two efforts having failed, a third method of hydrolysis was attempted. The feathers in this case were boiled with concentrated hydrochloric acid for

10 hours, and the acid was subsequently removed as far as possible by repeated vacuum-distillation, and most of the remainder, after neutralisation with sodium hydroxide, by treating the hydrolysate with its own volume of alcohol and leaving it in the ice-chest for a day. The filtrate was freed from tyrosine as before and the mixture dried out.

It is unnecessary to give the results; they repeat the finding of the other two hydrolysates, and the experiment was abandoned at this stage.

The only conclusion that can be drawn, therefore, is that the rat continues to grow, even when all the tyrosine is excluded from the diet. Whether this represents a real ability to synthesise the amino-acid for all purposes, or simply a power to use dietary phenylalanine, remains an open question.

SUMMARY.

Caseinogen hydrolysates, completely freed from tyrosine by means of tyrosinase, will support growth in young rats, whether supplemented with tyrosine or not.

Goose-feather keratin, however hydrolysed, and with the addition of tryptophan, tyrosine and phenylalanine, would not maintain young rats.

I am indebted to Sir F. G. Hopkins for his interest and encouragement.

REFERENCES.

Abderhalden (1913). *Z. physiol. Chem.* **83**, 444.
—— (1915). *Z. physiol. Chem.* **96**, 1.
—— and le Conte (1905). *Z. physiol. Chem.* **46**, 40.
Embden and Baldes (1913). *Biochem. Z.* **55**, 301.
Jackson, Sommer and Rose (1928). *J. Biol. Chem.* **80**, 167.
Lightbody and Kenyon (1928). *J. Biol. Chem.* **80**, 149.
Totani (1916). *Biochem. J.* **10**, 382.

# CLXI. A DIET WITHOUT CASEINOGEN FOR USE IN THE DETERMINATION OF VITAMIN A.

By RONALD SYDNEY MORGAN.

*From the Food Research Laboratory, Lever Brothers Limited, Port Sunlight, Cheshire.*

(*Received April 27th, 1934.*)

In the direct determination of vitamin A by a feeding test it is necessary to use a diet which is very deficient in vitamin A but which needs no other supplement to allow animals receiving it to grow at a rate of the same order as that of animals on a satisfactory diet of mixed foods such as is used for maintaining a breeding colony. The source of protein in the diets recommended for use in vitamin A determinations has always been caseinogen, a protein derived from milk, and therefore likely to be contaminated with vitamin A. Many workers consider it necessary to submit the caseinogen to a fairly prolonged heat treatment to destroy the vitamin A, but Coward *et al.* [1929; 1930; 1931] found that a diet containing heat-treated caseinogen as the source of protein was deficient in some factor other than vitamin A, so that after growth on the diet had ceased, supplements of cod-liver oil at any level failed to produce growth approaching the normal rate. They found it impossible to make any estimations of vitamin A from experiments in which a diet containing such caseinogen was used, because of the absence of a consistent gradation between the dosage of vitamin A and the growth response. A diet containing an untreated caseinogen ("light white casein"), however, was not deficient in the unknown factor; much better growth was obtained during the test period than could be obtained in experiments with the diet containing heated caseinogen, and the growth response was graded to the dosage of vitamin A so that satisfactory determinations of vitamin A could be made.

"Light white casein" contains an appreciable amount of butter fat (Culhane [1933] quotes determinations of 0·6 to 4·5 % with an average of 2·5 %), and it might be expected that this would supply sufficient vitamin A to interfere seriously with the development of symptoms of vitamin A deficiency; but in Coward's experience no such difficulty was met with, and it was found that "light white casein" defatted with alcohol and ether showed no advantage over the untreated caseinogen. Culhane [1933], however, concluded from a comparison of the effects of diets containing "light white casein," or heated caseinogen, on the development of ophthalmia that "light white casein" contains a considerable amount of vitamin A.

Bacharach [1933] found that the time required for the arrest of growth on a diet deficient in vitamin A, the weight reached before the growth arrest and the degree of recovery produced by doses of vitamin A were the same whether heated caseinogen or "light white casein" were used in the diet, but neither diet supplemented with vitamin A gave normal growth.

In our experience with a vitamin A-deficient diet similar in composition to that described by Coward, we were unable to obtain the early growth arrest

such as is obtained in her laboratory. Examinations of extracts from the livers of newly weaned rats failed to reveal the presence of sufficient quantities of vitamin A to account for the prolonged survival of rats on the deficient diet, and it seemed likely that our rats were obtaining significant quantities of vitamin A from the caseinogen. Unwilling to resort to the use of heated caseinogen because of the experience reported by Coward, and because A. F. Morgan [1931] has shown that strong heating damages proteins and makes them nutritionally unsatisfactory, we devised a diet containing no caseinogen, by using defatted meat meal and coconut meal to supply protein. The preliminary experiments were so encouraging that after some time the use of the caseinogen-containing diet was discontinued, and the new diet is now used in all our routine assays of vitamin A. Rats receiving the new diet show permanent growth arrest in about four weeks; they respond well to curative treatment with small doses of vitamin A, and the growth response is steeply graded to the dosage.

The present paper describes the technique for the determination of vitamin A as used in this laboratory, with a statistical treatment of the results so far available. The behaviour of rats on the new diet and the accuracy obtainable are compared with the results obtained with rats of the same colony fed on the diet containing caseinogen.

## Diets.

The rats used are albino rats of Wistar Institute strain. The breeding stock are fed on a cooked diet as previously described [Morgan, R. S., 1932]. The diet containing caseinogen was similar to that described by Coward *et al.* [1930]:

| | |
|---|---|
| "Light white casein" (B.D.H.) ... ... | 15 % |
| Starch ... ... ... ... ... | 73 % |
| Dried yeast ... ... ... ... | 8 % |
| Salt mixture (Steenbock's No. 40) ... | 4 % |
| Vitamin D ... ... ... ... ... | 8 units per week |

Originally the starch was supplied as raw rice starch and the diet was cooked with water to a stiff jelly before feeding. Later the raw starch was replaced by ground flaked rice (cooked over rollers) and the diet was fed dry. This change did not affect the performance of the rats.

The diet without caseinogen is as follows:

| | |
|---|---|
| Ground flaked rice ... ... ... | 48 % |
| Coconut meal ... ... ... ... | 30 % |
| Defatted meat meal ... ... ... | 10 % |
| Dried yeast ... ... ... ... | 8 % |
| Salt mixture (Steenbock's 40) ... ... | 4 % |
| Vitamin D ... ... ... ... ... | 8 units per week |

A large stock of dried meat meal was specially prepared. It was dried with a minimum exposure to heat in order to avoid damage to the protein. Quantities were defatted from time to time as required in a small solvent-extraction plant.

## Conduct of experiments.

Litters are not reduced in size and they are weaned on to the deficient diet at 23 days of age unless, as occasionally happens, the average weight is then less than 25 g., when they are left with the mother for a further week. All the rats of any one litter intended for test are started on experiment on the same day. Pairs of rats are selected of the same sex and as nearly as possible of the same

weight; one of each pair receives a daily dose of the test substance and the other receives a comparable dose of the international carotene standard. Arachis oil is the solvent used for dissolving the carotene standard and for diluting oils to be assayed. It is obtained direct from our own refinery; on receipt, quinol is added, and the oil is allowed to stand at room temperature for a day with

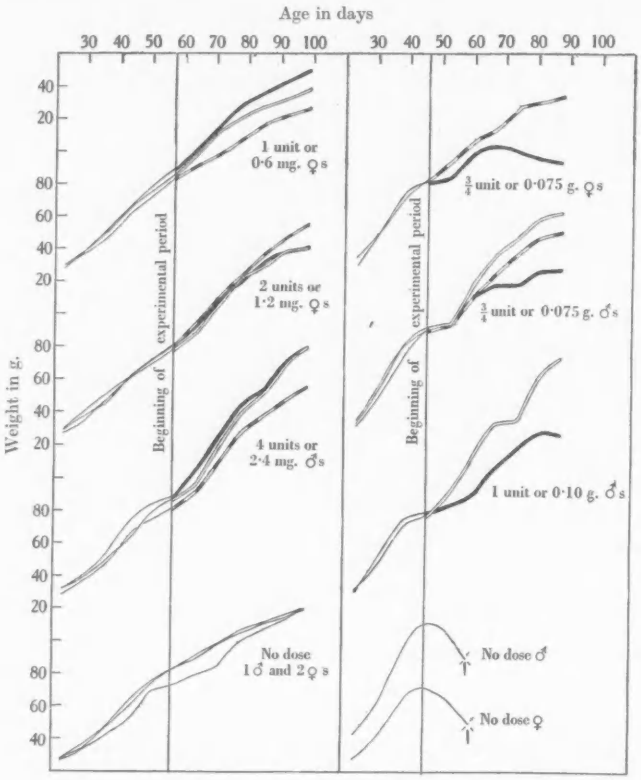

Age in days
30 40 50 60 70 80 90 100
30 40 50 60 70 80 90 100
Weight in g.
Beginning of experimental period
Beginning of experimental period
1 unit or 0·6 mg. ♀s
2 units or 1·2 mg. ♀s
4 units or 2·4 mg. ♂s
No dose 1♂ and 2♀s
¾ unit or 0·075 g. ♀s
¾ unit or 0·075 g. ♂s
1 unit or 0·10 g. ♂s
No dose ♂
No dose ♀
carotene standard
red palm oil 1
red palm oil 2
carotene standard
butter 1
butter 2

Fig. 1. Typical growth curves of litters from vitamin-A tests showing the difference between the behaviour of rats on the diet with casein and that of rats on the diet with meat meal and coconut meal as sources of protein.

occasional shaking. Under these conditions the oil absorbs about 0·05 % quinol. It is then stored in a refrigerator. A strong solution of the carotene standard is prepared by dissolving a weighed quantity in a little benzene, adding arachis oil and evaporating the benzene under reduced pressure. Sub-dilutions of this solution for the purpose of dosing are prepared from time to time. The stability of carotene dissolved in this way is satisfactory. Judging by Lovibond

matches on dilutions with chloroform, we found no significant loss of carotene from a solution after storage in a refrigerator for six months.

In the experiments from which the data presented in this paper have been taken, a test period of five weeks has been used, but the animals have been continued on test for a further week and the growth has been expressed as the average of the growths made in 4, 5 and 6 weeks.

In the experiments with the diet containing caseinogen, animals would sometimes still be growing slowly after they had been receiving the deficient diet for 13 weeks, though others might have died much earlier. It was impossible to wait for a permanent arrest of growth before beginning a test, because this was sometimes not obtained, and we adopted the practice of starting the rats of a litter on test after about 5–7 weeks on the deficient diet and keeping some animals as negative controls. Usually these negative controls would survive the test period and make some growth, but it was nearly always less than the growth given by the lowest dose of standard or test substance.

In experiments with the diet without caseinogen, the rats cease to grow in about four weeks from weaning. They are started on test as soon as the growth rate has begun to slacken; this happens for all the rats of a litter at about the same time, and incipient xerophthalmia is usually apparent throughout the litter. It is not advisable to wait for a decline in weight, for many of the rats may then be lost; they may fail to respond to quite large doses of vitamin A and die within a few days of the beginning of the test; or even if they survive, their growth rate may be determined rather by the seriousness of their condition at the beginning of the test than by the dosage of vitamin A, so that even a positive control receiving about 20 units per day may grow at a very subnormal rate.

The difference between the behaviours of rats on the diets with and without caseinogen is shown in Fig. 1 which gives the growth curves of two typical litters.

## Relation between growth-response and dosage; interpretation of assays.

On both the vitamin A-deficient diets that have been used in this laboratory there is a definite increase in the growth-response with increase in the dosage of vitamin A, but this increase is much more striking in the experiments with the diet without caseinogen than in the experiments with the diet with caseinogen.

*Diet with caseinogen.* For the interpretation of experiments when the diet with caseinogen was used sufficient data were not available to construct a curve showing the average relation between growth-response and dosage of the standard preparation. Coward *et al.* [1930; 1931] showed that for their colony, using practically the same vitamin A-deficient diet, the relation was given by the equation:

$$\text{growth-response} = c + k \log \text{dose},$$

and a similar relation has been assumed in the interpretation of our experiments.

Since it is our custom to feed the test substance and the standard at at least three levels of dosage, it was possible to calculate our own values of the constant $k$ in the above equation from the average difference between the growth-responses of two animals of the same litter receiving doses of the same substances in the ratio of 2 to 1. These average values were as follows:

Difference in growth-response (average at 4, 5 and 6 weeks) corresponding to a dose ratio of 2 to 1.

Bucks (37 values) 12·05 g. $k = 12{\cdot}05/\log 2 = 40{\cdot}0$
Does (54 values) 8·4 g. $k = 8{\cdot}4/\log 2 = 27{\cdot}9$

If the growth-response is measured as the actual growth in 5 weeks instead of as the average in 4, 5 and 6 weeks, the values of $k$ become: bucks 37·6, does 26·1. These values would be expected to be somewhat lower than those obtained by Coward *et al.*, because in nearly all our experiments the negative controls made some growth, and if this is attributed to vitamin A in the diet, it follows that when the doses given to two rats are in the ratio of 2 to 1 the total vitamin A received will be in a lower ratio, *viz.*: $(2+x)/(1+x)$, where $x$ is the amount of vitamin A supplied by the diet.

The values of $k$ given by Coward *et al.* [1931] were originally: bucks 50·3, does 27·4, but with the accumulation of further data, they were modified [Coward, 1933] to: bucks 45·81, does 27·0.

*Diet without caseinogen.* The smallest dose of the standard carotene that we use is 0·5 unit. This is often insufficient to allow the animals to survive the full test period, but some growth-response is usually given at the beginning of the test. In such a case the growth-response is recorded up to the maximum weight obtained. This practice is justified by the observation that when a substance has been fed at two levels both failing to allow full survival, the maximum growth-response is still graded to the dosage.

The inter-litter variation in growth-response is very great; the response to a dose of 2 units in one litter may exceed that of the positive controls in another litter. The international carotene standard has not yet been fed to a sufficient number of rats at a sufficient variety of levels to allow a satisfactory average curve relating growth-response and dosage to be drawn. The average values so far obtained are given in Table I.

Table I. *Average values of the growth-response produced by doses of the international carotene standard.*

| | Bucks | | Does | |
|---|---|---|---|---|
| Dose | Growth-response in g. averaged at 4, 5 and 6 weeks | No. of animals | Growth-response in g. averaged at 4, 5 and 6 weeks | No. of animals |
| 0·5 unit | 23·4 | 12 | 24·8 | 26 |
| 0·75 unit | 45·7 | 13 | 31·4 | 22 |
| 1 unit | 55·3 | 64 | 42·3 | 43 |
| 2 units | 76·7 | 53 | 59·6 | 7 |
| Positive controls (about 20 units) | 94·5 | 22 | 60·3 | 12 |

Soon after the new diet was first introduced, however, it became evident that the difference between the growth-responses produced by 0·5 unit and 1 unit was greater than the difference between the growth-responses produced by 1 unit and 2 units. The average values of the difference in growth-responses between animals of the same litter receiving doses of the standard, or of substances undergoing assay, in the ratio of 2 to 1 have been calculated separately for the higher levels and the lower levels of dosage. They are as follows:

| | Bucks | No. of values | Does | No. of values |
|---|---|---|---|---|
| Higher levels | 22·8 | 45 | 11·0 | 9 |
| Lower levels | 35·9 | 19 | 16·7 | 27 |

It is evident therefore that the growth-response does not increase so rapidly with the dosage as is required by the equation

$$\text{growth} = c + k \log \text{dose}$$

and that the relation will be better expressed by some such equation as

$$(\text{growth})^n = c + k \log \text{dose},$$

where $n$ is greater than unity.

For the purpose of interpreting assays, $n$ has been taken as 2, *i.e.* the square of the growth is taken as proportional to the logarithm of the dose. The average values of the difference in the squares of the growth-response between animals of the same litter receiving doses in the ratio of 2 to 1 are:

| | Bucks | Does |
|---|---|---|
| Higher levels | 3100 | 1050 |
| Lower levels | 3590 | 1248 |

and thus the values for the two levels agree with each other much more closely than when the differences in the growth-responses themselves are considered.

Considering all values together, including the values that can be derived from the difference in the squares of the growth-response between animals receiving doses in the ratio $\frac{1}{2}$ to $\frac{3}{4}$, or $\frac{3}{4}$ to 1, the average values of the increment in the square of the growth produced by doubling the dose are:

| | |
|---|---|
| Bucks (70 values) | 3235 |
| Does (46 values) | 1310 |

By means of these values, the average growth-responses produced by various doses of the standard (Table I) can be averaged at 1 unit as 54·7 for the bucks and 42·4 for the does. The full equations relating growth to dosage in units of standard are then calculated as:

$$\text{Bucks } (\text{growth})^2 = 2992 + 10{,}730 \log \text{dose}$$
$$\text{Does } (\text{growth})^2 = 1798 + 4{,}350 \log \text{dose}.$$

The curves relating growth to dosage given by these equations are plotted in Fig. 2, which shows also the actual points taken from Table I. These curves are

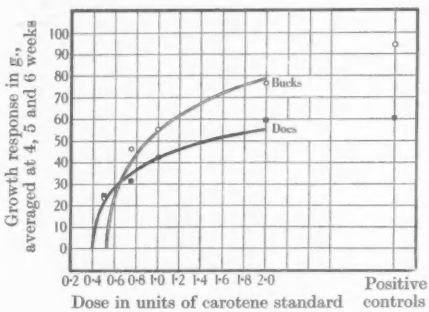



Fig. 2. Relation between growth response and dosage of vitamin A in vitamin A tests. The points are the average values given in Table I.

not designed to fit the points; their slopes have been determined from the average values of the increment in the square of the growth produced by doubling the dose, and the average values of the growths produced by various doses of the standard preparation have been introduced merely to give the positions of the curves. Two features of the curves require some comment. (i) The curve for bucks gives an imaginary value for the growth of rats receiving 0·5 unit of

standard, while the average growth given in Table I is 23·4 g. Even if adequate data were available, however, it would be difficult to calculate a satisfactory average value of the growth response produced by 0·5 unit, since when a rat receiving 0·5 unit loses weight and dies, it is impossible to tell whether the dose was insufficient for the rat, or whether it was in such a weakened condition that it would have died even if the dose had been 2 or 4 times as large. (ii) Both curves show the growth as increasing indefinitely with increasing dosage, while it is evident that an upper limit is set to the growth by the maximum growth capacity of the rat; but if the curves were actually limited by the average growth of positive controls, which is approximately equal to normal growth, many individual values obtained would lie above the curve at all levels of dosage. In practice the relation between growth and dosage given by the curve holds satisfactorily from slight growth up to the growth produced by doses of about 4 units; it is not claimed to be valid for doses above about 4 units.

The method of interpreting an assay is made clear by an example (Table II).

Table II. *Assay of a sample of cod-liver oil, blue value = 10·5, blue value via unsaponifiable matter = 24·2.*

| Litter | Dose units | Growth in g. Bucks | Does | Dose mg. | Growth in g. Bucks | Does |
|---|---|---|---|---|---|---|
| 822 | 0·5 | 14·7 | — | 1·11 | 46 | — |
| 824 | 1 | — | 43 | 2·22 | — | 37 |
| | 2 | 88·3 | — | 4·44 | 97 | — |
| 826 | 1 | 57·7 | — | 2·22 | 73·3 | — |
| 828 | 0·75 | — | 21 | 1·67 | — | 40 |
| 830 | 1 | — | 47·3 | 2·22 | — | 59 |
| | 2 | 81·7 | — | 4·44 | 89·3 | — |
| 832 | 0·5 | — | 19·7 | 1·11 | — | 35·3 |
| | 1 | 67·3 | — | 2·22 | 88 | — |
| | 2 | 88·7 | — | 4·44 | 93·3 | — |
| 834 | 1 | — | 30 | 2·22 | — | 57 |
| 839 | 1 | 34·3 | — | 2·22 | 79·7 | — |
| | 2 | 56·3 | — | 4·44 | 84 | — |
| 841 | 0·75 | — | 43·3 | 1·67 | — | 59·7 |
| | 1 | — | 57·7 | 2·22 | — | 67 |
| 843 | 0·75 | — | 42·7 | 1·67 | — | 57·7 |
| | 1 | 62·3 | — | 2·22 | 75 | — |
| | 2 | 82 | — | 4·44 | 100·3 | — |

The pair differences of the squares of growth, *i.e.* the differences between the squares of figures on the same horizontal line in the above table are:

Cod-liver oil *minus* standard.

| | Bucks | Does |
|---|---|---|
| | 1900 | − 480 |
| | 1612 | 1159 |
| | 2044 | 1244 |
| | 1299 | 670 |
| | 3215 | 2349 |
| | 1881 | 1689 |
| | 5176 | 1160 |
| | 3886 | 1506 |
| | 1656 | — |
| | 3336 | — |
| Averages | 2601 | 1162 |

These mean pair differences correspond to dose ratios calculated from the equations relating growth response to dosage thus:

Bucks: Dose ratio (2·22 mg. oil)/(1 unit standard)
= antilogarithm of 2601/10,730
= antilogarithm of 0·242
= 1·746;

*i.e.* 2·22 mg. of oil = 1·746 units of standard, and the assay according to the bucks is 785 units per g.:

Does: Dose ratio (2·22 mg. oil)/(1 unit standard)
= antilogarithm of 1162/4350
= antilogarithm of 0·267
= 1·849;

*i.e.* the assay according to the does is 830 units per g. The average assay is calculated from the weighted average of the logarithms of the dose ratios (0·242 for the 10 pairs of bucks and 0·267 for 8 pairs of does), but, for reasons given below, 1 pair of bucks is considered equivalent to 2 pairs of does. The weighted average of the logarithms of the dose ratios is thus:

$(0{\cdot}242 \times 20 + 0{\cdot}267 \times 8)/28 = 0{\cdot}249$
Dose ratio = 1·774
Assay = 800 units per g.

### Calculation of errors.

*Diet with caseinogen.* Coward [1932; 1933] calculated the average accuracy of a determination of vitamin A from the variation within a group of rats each of which received the same dose. The standard deviation from the mean growth in a five-week test was given in the later paper as 15·37 g. for bucks and 11·93 g. for does. In a test in which the growth-response produced by a substance of unknown potency is compared with that produced by the standard, the standard deviation of the difference between two observations is required. This value would be expected to be $15{\cdot}37\sqrt{2} = 21{\cdot}7$ g. for bucks and $11{\cdot}93\sqrt{2} = 16{\cdot}9$ g. for does. Three times the probable error of the difference between two means in an experiment in which 10 animals received the standard and 10 received the test substance can then be calculated as:

$2 \times 21{\cdot}7/\sqrt{10} = 13{\cdot}7$ g. for bucks,
$2 \times 16{\cdot}9/\sqrt{10} = 10{\cdot}7$ g. for does.

From Coward's equations relating growth to dosage, these errors correspond to dose ratios of:

antilog 13·7/45·81 = 1·99 for bucks,
antilog 10·7/27·0 = 2·49 for does,

*i.e.* the percentage errors corresponding to three times the probable error in a determination of vitamin A with two groups of 10 animals each are

+99 or −50 % if all the animals are bucks, or
+149 or −60 % if all the animals are does.

In our own experiments using practically the same diet as that used by Coward *et al.*, in which we obtained the same order of increase in growth with

increase of dosage, the average values of the difference between the growth of two animals of the same litter receiving doses in the ratio of 2 to 1 were

for the bucks 12·05 g. (37 values), standard deviation = 13·02 g.,
for the does 8·4 g. (54 values), standard deviation = 10·87 g.

These standard deviations should be about the same as the standard deviation of the difference between two observations. Three times the probable error of the mean pair difference of ten pairs is calculated as

$2 \times 13{\cdot}02/\sqrt{10} = 8{\cdot}25$ g. for bucks,
$2 \times 10{\cdot}87/\sqrt{10} = 6{\cdot}89$ g. for does.

Taking values of $k$ in the equations relating growth to dosage as 40·0 for bucks and 27·9 for does, these errors correspond to dose ratios of

antilog $8{\cdot}25/40{\cdot}0 = 1{\cdot}61$ for bucks,
antilog $6{\cdot}89/27{\cdot}9 = 1{\cdot}77$ for does,

*i.e.* the percentage errors corresponding to three times the probable error in a determination of vitamin A with 10 pairs of animals are

+61 or −38 % if all the animals are bucks, or
+77 or −44 % if all the animals are does.

These values are very much smaller than those calculated from Coward's values of the standard deviation of the mean growth of a group of animals receiving the same dose of vitamin A. The difference between the two calculations represents mainly the gain in accuracy that is to be expected by confining consideration to litter-mate pairs started on test on the same day. Admittedly the second calculation is based on fewer data than the first, but in view of the elimination of inter-litter variation it would be expected to give much lower values.

*Diet without caseinogen.* Since, in the experiments with the new diet containing meat meal and coconut meal as sources of protein, it has been found that the growth is graded much more steeply to the dosage than in the experiments with the diet containing caseinogen, it would be expected that a further reduction of error would be obtained. The average differences between the squares of the growth of animals of the same litter receiving doses in the ratio of 2/1 were calculated as

for bucks 3235 (70 values), standard deviation = 2100,
for does 1310 (46 values), standard deviation = 1005.

From the equations relating the square of the growth to the logarithm of the dosage, given above, the values of the dose ratio corresponding to three times the probable error of the mean pair difference in an experiment with 10 pairs of animals are calculated as

for bucks, antilog $2 \times 2100/10{,}730\sqrt{10} = 1{\cdot}33$,
for does, antilog $2 \times 1005/4350\sqrt{10} = 1{\cdot}40$,

*i.e.* the percentage errors corresponding to three times the probable error are

+33 or −25 % for 10 pairs of bucks, or
+40 or −29 % for 10 pairs of does.

A second calculation of the errors to be expected in experiments with the new diet has also been made from the mean variance of the pair difference determined in each test.

For the bucks, the sum of the squares of the deviations from the mean pair difference in each test was 374,320,000; the number of pairs was 193 and the number of tests 38. The standard deviation is given as

$$\sqrt{\{374320000/(193-38)\}}=1554,$$

from which the percentage error corresponding to 3 times the probable error is calculated as

$$+24 \text{ or } -19\ \% \text{ for 10 pairs of bucks.}$$

For the does, the sum of the squares of the deviations from the mean pair difference in each test was 103,120,000; the number of pairs was 149 and the number of tests 35. The standard deviation is therefore

$$\sqrt{\{103120000/(149-35)\}}=950,$$

and the percentage error corresponding to three times the probable error is

$$+37 \text{ or } -27\ \% \text{ for 10 pairs of does.}$$

These errors are smaller than the errors calculated from the variation in the difference between the squares of the growth of litter-mates receiving doses in the ratio of 2/1. Two factors operate in this reduction of the calculated error: (i) in the last calculation the variation is in the difference between litter-mates selected to be as nearly as possible of the same weight at the beginning of the experimental period, while in the first the variation is between litter-mates not so selected, and (ii) in the second calculation the curve relating growth to dosage is assumed to be absolutely correct, *i.e.* no account is taken of error introduced in the interpretation of an assay by reference to a curve which may not be strictly applicable to the group of rats under consideration. Even if the curve were constructed from data obtained from several thousands of rats so that it represented very accurately the mean behaviour, some error from this source might still enter an assay; for just as some litters grow more rapidly than others, so the growth of some may be more steeply graded to dosage than that of others. The magnitude of the error derived from this source will depend entirely on the magnitude of the mean difference between the growths produced by the test substance and the standard, from which the assay will be calculated. If this difference is zero, *i.e.* if the comparative doses of test substance and standard produce equal average growth, the error is eliminated, because the standard curve of relation between growth and dosage is not required. The relation between growth and dosage given in this paper is, for bucks:

$$(\text{growth})^2=c+10{,}730 \log \text{dose};$$

if for a certain assay group of rats the true relation should be

$$(\text{growth})^2=c+9000 \log \text{dose},$$

the error introduced by interpreting the assay by reference to the standard relation would be 0 for a dose ratio test-substance/standard = 1, but if the true dose ratio were 2/1, an error of $-11$ or $+12$ % would be introduced in the interpretation of the assay, and if the true dose ratio were 4/1 the error would be $-20$ or $+25$ %.

The second calculation of errors therefore gives the expected error in a test in which the comparative doses of the test substance and the standard produce nearly equal growth. The first calculation gives the expected error in an assay in which the doses of test substance supply about twice as much vitamin A as the comparative doses of standard. The above calculations of the errors expected in

a determination of vitamin A are summarised in Table III. The number of pairs of bucks required to give an assay of the same accuracy as that obtained with 10 pairs of does has been calculated from the limits of error given by each method, and the figures are given in the last column.

Table III. *Summary of the calculations of the errors expected in vitamin A determinations.*

| Diet | Basis of calculation | Upper limit of error (%) corresponding to 3 times the probable error in an assay in which 10 rats receive the test substance and 10 receive the standard | | No. of pairs of bucks required to give an assay of the same accuracy as that given by 10 pairs of does |
|---|---|---|---|---|
| | | Bucks | Does | |
| Diet with caseinogen | The variation within groups of rats receiving the same dose of vitamin A [Coward, 1933] | 99 | 149 | 5·7 |
| „ | The variation in the growth difference between litter-mates receiving doses in the ratio 2/1 | 61 | 77 | 6·9 |
| New diet without caseinogen | The variation in the difference between the squares of growth of litter-mates receiving doses in the ratio 2/1 | 33 | 40 | 7·1 |
| „ | The variation in the difference between the squares of growth of litter-mate pairs, one of which receives the test substance and the other the standard | 24 | 37 | 4·4 |

Coward [1932] has already pointed out that to get equal degrees of accuracy, about twice as many does as bucks must be used in a test. Our own figures confirm this finding. For this reason, in the interpretation of an assay in which both bucks and does have been used, one pair of bucks is considered equivalent to two pairs of does. For the same expenditure of labour, however, greater accuracy may be obtained by using bucks only in vitamin A tests; litters at weaning may be separated into bucks and does, the bucks being taken for vitamin A tests and the does for some other purpose, such as vitamin D tests, for which they are as suitable as bucks.

Coward [1933] has reported that the error of an assay with a 3-week experimental period is only slightly greater than that of an assay with a 5-week experimental period. The relation between the errors of 3-week and 5-week assays has also been calculated from the records of experiments in which the new diet without caseinogen has been used. The mean difference between the squares of the growth-responses in three weeks of two rats receiving doses in the ratio 2/1, is

for bucks: 1785, standard deviation 1332,
for does: 606, standard deviation 563.

The equations relating growth-response to dosage are

$$\text{Bucks: (growth)}^2 = 1521 + 5930 \log_{10} \text{dose},$$
$$\text{Does: (growth)}^2 = 900 + 2013 \log_{10} \text{dose}.$$

The percentage error, corresponding to 3 times the probable error, of a test with 10 pairs of animals, is calculated from these figures as +39 for bucks, or +50 for does. By comparison with the values calculated in the same way from the growth-responses averaged at 4, 5 and 6 weeks, it is found that 14 pairs of rats in a 3-week experiment will give an assay of about the same accuracy as that given by 10 pairs in (4, 5, 6)-week experiment. By a similar calculation it is found that if the growth-response in 6 weeks is considered, the number of pairs of rats required to give the same accuracy is 10·6.

## Discussion.

The diet described in this paper, with meat meal and coconut meal as sources of protein, is in our experience very satisfactory for use in the determination of vitamin A. It is so deficient in vitamin A that animals receiving it show a permanent growth arrest in about 4 weeks, while animals of the same colony receiving a diet with 15 % of "light white casein" may continue to grow for 10 weeks or more; and when supplemented with vitamin A, it allows growth of the same order as that of rats of the breeding colony receiving the stock diet. This is seen by the performance of positive controls given about 20 units of vitamin A daily (1 drop of oil of 56 blue value).

*Bucks.*

22 positive controls, average growth-response = 94·5 g.
Average weight at beginning of experimental period = 76 g.
Average age at beginning of experimental period = 49 days.

From the average growth curves of rats of the breeding colony, the growth of bucks averaged at 4, 5 and 6 weeks after they attain a weight of 76 g. (at 41 days) is 91 g.

*Does.*

12 positive controls, average growth-response = 60·3 g.
Average weight at beginning of experimental period = 71 g.
Average age at beginning of experimental period = 49 days.

From the average growth curves of rats of the breeding colony, the growth of does averaged at 4, 5 and 6 weeks after they attain a weight of 71 g. (at 43 days of age) is 62 g.

Since the diet only needs to be supplemented with vitamin A to give normal growth (at least for the periods considered here), it does not appear to be deficient in any factor essential to growth, other than vitamin A. The only ingredient of the diet originally in use that is omitted from the new diet is "light white casein," and since the new diet produces a much earlier development of symptoms of vitamin A deficiency than did the original diet, it appears likely that the difference is indeed due to the presence of vitamin A in "light white casein" in sufficient amount to hinder the development of vitamin A deficiency in animals of our stock.

The observation that the difference in growth between animals receiving doses in the ratio of 2/1 is greater in the experiments using the diet without caseinogen than it is in the experiments using the diet with caseinogen, also supports the view that the diet without caseinogen is more deficient in vitamin A than the other; for if two rats receive doses in the ratio of 2/1 and they also obtain some vitamin A from the diet, the ratio of the total amounts of vitamin A received will be less than 2/1, and therefore the growth difference will be less than if the diet had supplied no vitamin A. In our experiments using the diet with caseinogen the negative controls usually showed some growth, but the

does survived the full experimental period more often than the bucks; a growth of 25 g. during the experimental period was quite usual, and generally the negative controls resembled rats receiving 0·5 unit daily in the experiments using the diet without caseinogen. If it is assumed that the caseinogen was actually supplying about 0·5 unit of vitamin A daily, the growth-responses that would be produced by doses of the standard may be calculated from our equations relating growth-response to total vitamin A (Fig. 2) as follows:

Table IV. *Relation between growth-response and dosage expected if diet supplies about 0·5 unit of vitamin A daily.*

| Dose | Total vitamin A received including 0·5 unit daily from the caseinogen | Growth-response calculated from the curves given in Fig. 2 | |
|---|---|---|---|
| | | Bucks (g.) | Does (g.) |
| No dose | $\frac{1}{2}$ unit | 0 | 22·1 |
| $\frac{1}{8}$ unit | $\frac{5}{8}$ ,, | 28·2 | 30·1 |
| $\frac{1}{4}$ ,, | $\frac{3}{4}$ ,, | 40·7 | 35·4 |
| $\frac{1}{2}$ ,, | 1 ,, | 54·7 | 42·4 |
| 1 ,, | $1\frac{1}{2}$ units | 69·9 | 50·7 |
| 2 units | $2\frac{1}{2}$ ,, | 85·3 | 59·4 |

From the figures in Table IV it is seen that using a diet supplying about 0·5 unit of vitamin A daily, it would be expected that within the range 0·25 to 2 units the growth-response would be related to the dosage (excluding the vitamin A obtained from the diet) by

$$\text{growth} = c + k \log \text{dose}.$$

The increments in growth-response produced by successively doubling the dose from 0·25 unit to 2 units are approximately constant and are calculated from the above figures as 14·0 g., 15·2 g. and 15·4 g. for bucks, and as 7·0 g., 8·3 g. and 8·7 g. for does. These values are of the same order as those actually obtained in our experiments with the caseinogen diet (12 g. for bucks and 8·4 g. for does).

If 15 % of caseinogen in the diet supplies about 0·5 unit of vitamin A daily to rats eating about 10 g. of food per day, the caseinogen should contain $\frac{1}{3}$ unit per g. Summer butter-fat, according to our assays, contains about 18 to 25 units per g., and the amount of butter-fat required to be present in caseinogen to account for this potency would be 1·4 %. Culhane, as quoted above, gives the average fat content of "light white casein" as 2·5 %, so that a fair margin for loss of vitamin A from the fat during the manufacture and storage of the caseinogen can be allowed for.

Apart from the question of the composition of the diet, it is suggested that the following conditions should be observed for the most accurate assay of vitamin A.

(i) The carotene standard and oils to be tested should be dissolved in an oil in which they are stable over periods of several months. Arachis oil obtained direct from the refinery and left to stand over quinol fulfils this condition.

(ii) The growth of rats receiving the test substance should be compared with that of rats receiving the carotene standard, each rat being compared with another of the same litter and as nearly as possible of the same weight, started on test on the same day. If one rat of such a pair dies at the beginning of the test period, its control is not considered in interpreting the assay.

(iii) The comparative doses of the test substance and the carotene should be selected, if necessary after a preliminary trial, to give nearly equal average growth-responses.

SUMMARY.

A vitamin A-free diet not containing caseinogen has been used in the routine assay of vitamin A. This diet unsupplemented with vitamin A produces a permanent arrest of growth in about four weeks, and growth at the normal rate can then be restored by the addition of vitamin A. Animals of the same colony given a diet with 15 % of "light white casein" as the source of protein may grow for 10 weeks or more, before showing a permanent growth arrest. The difference is attributed to the presence of vitamin A in the "light white casein."

When the above diet with defatted meat meal and coconut meal as sources of protein is used, the growth is graded steeply to the dosage.

From a review of the results so far obtained it is concluded that the growth-response is related to the dosage of vitamin A (in international units) approximately by the equations:

The square of the growth-response $= 2992 + 10{,}730 \log_{10}$ dose (for bucks),

or $1798 + 4{,}350 \log_{10}$ dose (for does).

The growth-response is taken as the growth in g. averaged at 4, 5 and 6 weeks after the beginning of the test period. In carrying out an assay, the test substance and the carotene standard are fed at three or four levels, and the growth of each rat receiving a dose of test substance is compared with that of a rat receiving the standard, of the same litter, the same age and as nearly as possible the same initial weight, started on test at the same time.

From the results so far available it is calculated that the limits of error corresponding to three times the probable error (*i.e.* the error expected once in 22 tests) in an assay with 10 pairs of rats are:

(i) If the comparative doses of standard and test substance supply nearly equal amounts of vitamin A

$-19$ to $+24$ % for bucks,
$-27$ to $+37$ % for does.

(These values are calculated from the results of tests with 342 pairs of rats.)

(ii) If the comparative doses of standard and test substance supply amounts of vitamin A in the ratio of about 2/1

$-25$ to $+33$ % for bucks,
$-29$ to $+40$ % for does.

(These values are calculated from the results of tests with 116 pairs.) These errors are much lower than those obtained in assays with a diet containing caseinogen.

Coward's findings [1932; 1933] that about twice as many does as bucks are required to obtain assays of the same degree of accuracy, and that the error of an assay with an experimental period of 3 weeks is only slightly greater than that of an assay with an experimental period of 5 weeks, are confirmed.

Arachis oil is the solvent used for dissolving the carotene standard and for diluting oils to be assayed; it is obtained direct from the refinery and allowed to stand over quinol. No loss of carotene dissolved in this solvent and stored in a refrigerator has been observed during six months.

REFERENCES.

Bacharach (1933). *Biochem. J.* **27**, 5.
Coward (1932). *Biochem. J.* **26**, 691.
—— (1933). *Biochem. J.* **27**, 445.
Coward, Key, Dyer and Morgan, B. G. E. (1930). *Biochem. J.* **24**, 1952.
—— —— —— —— (1931). *Biochem. J.* **25**, 551.
—— —— Morgan, B. G. E. and Cambden (1929). *Biochem. J.* **23**, 913.
Culhane (1933). *Biochem. J.* **27**, 69.
Morgan, A. F. (1931). *J. Biol. Chem.* **90**, 771.
Morgan, R. S. (1932). *Biochem. J.* **26**, 1144.

# CLXII. RAPID METHODS FOR THE QUANTITATIVE DETERMINATION OF TOTAL PROTEIN AND NON-PROTEIN NITROGEN IN HUMAN AND COW'S MILK.

By LESLIE DOUGLAS SCOTT.

*From the Biochemical Department, Charing Cross Hospital, Institute of Pathology.*

(*Received June 14th, 1934.*)

Most methods for the determination of protein in milk depend on the Kjeldahl analysis of the milk in the presence of the lactose and non-protein nitrogen, because there is no known convenient milk-protein precipitant that allows of two complete washings of the protein precipitate without loss of protein. The nitrogen determination is thus always too high and the presence of the non-protein nitrogen and the relatively large amount of lactose adds to the material to be oxidised. Moreover the excessive carbonisation and resulting loss of sulphur trioxide caused thereby are responsible for the formation of a cloudy digest unsuitable for direct nesslerisation. Time-consuming procedures such as distillation or aeration have therefore to be employed.

Likewise, the recent formaldehyde titration method proposed by Pyne [1932], although rapid and simple, fails to give a true total protein content of milk, in that the formaldehyde titration factor used is obtained by standardisation against the total nitrogen as estimated by the Kjeldahl process.

The method presented here overcomes these difficulties by double precipitation of the protein with zinc hydroxide, the supernatant fluid being separated by centrifuge, and, if necessary, retained for the estimation of the non-protein nitrogen. The protein is subjected to a micro-Kjeldahl digestion employing a modified Folin and Denis [1916] reagent in which the proportion of catalyst and the concentration of the digestion mixture are raised. This overcomes the inhibitory action of the fat which is partly precipitated and adsorbed on the protein precipitate, as well as the resistant physical character of the milk protein itself. The clear digest which results is diluted and directly nesslerised. By using the special digestion mixture direct determination of the non-protein nitrogen is possible, obviating the necessity of introducing elaborate procedures such as that devised by Denis and Minot [1919] to remove first lactose, fat and other interfering substances.

The present method as finally evolved provides a technique which is very convenient for the requirements of a routine laboratory, making possible a quantitative determination of proteins in less than 30 minutes.

It is necessary to emphasise the importance of using only fresh samples for analysis. Where a delay is inevitable, sodium fluoride to the extent of 1 % must be added.

*Reagents.*

(1) 0·45 % zinc sulphate.
(2) *N*/10 and *N*/100 NaOH.
(3) 40 % NaOH.
(4) *N*/100 $H_2SO_4$.
(5) Standard ammonium sulphate 0·494 g. per litre. 10 ml. contain 1·04 mg. nitrogen.
(6) Digestion mixture:
50 ml. of 10 % copper sulphate;
300 ml. of syrupy (85 %) phosphoric acid;
100 ml. of pure nitrogen-free sulphuric acid.
(7) Nessler-Folin reagent [Folin and Wu, 1919].

*Method.*

To 10 ml. of 0·45 % zinc sulphate in a centrifuge-tube graduated to hold about 15 ml., add 1 ml. of human or 0·5 ml. of cow's milk. Mix well by inserting a rubber stopper or by placing the thumb over the opening of the tube and shaking vigorously, and then add 2 ml. of *N*/10 sodium hydroxide. Mix again, and after standing for about one minute or until the protein flocculates centrifuge at high speed (over 2000 r.p.m.) for 5 minutes. The supernatant fluid may be slightly opalescent with specimens of human milk, if the fat content is above normal, and this is an added advantage as the excess of fat is removed with the supernatant fluid and therefore does not retard the digestion process by heavy carbonisation. Carefully pour off the supernatant fluid, and add another 10 ml. of zinc sulphate solution. Mix vigorously to obtain a uniform emulsion of precipitated protein and add 1 ml. of the *N*/10 sodium hydroxide. After thorough mixing, again centrifuge at high speed for 5 minutes. Pour off the supernatant fluid and transfer the precipitated protein to a round-bottomed clear silica flask of 50 ml. capacity, using exactly 10 ml. of distilled water for transferring. It is convenient to have the distilled water in a graduated cylinder. Measure approximately 5 ml. of water into the centrifuge-tube, insert a rubber stopper and shake vigorously. Carefully remove the stopper and transfer the extract to the silica flask. Repeat the above procedure using 3 ml. and finally 2 ml. of water, transferring all the washings to the silica flask. This procedure removes all the remaining precipitate adhering to the sides of the centrifuge-tube and the rubber stopper, with the exception of a little precipitated fat. The total volume of wash-fluid in the silica flask should not be greater than 10 ml. Finally add 5 ml. of digestion mixture and proceed with the digestion carefully so as to obtain a clear digest.

Ordinarily the digestion is carried out in a fume chamber, or better under a hood to which suction is applied, as the fumes by this method are exceedingly heavy. With the flask somewhat inclined, boil vigorously over a micro-burner, using a long thin flame of about 4 in. in height which just touches the flask and spreads out slightly over the round bottom. To prevent bumping keep the flask moving so that the fluid spins round vigorously. At the first signs of boiling, heat the flask very cautiously, removing the flame if necessary, until the initial foaming subsides. This ceases after only a few seconds. Vigorous boiling is continued until the organic matter begins to carbonise. This usually occurs about 2 minutes after commencement of boiling. It is now necessary to place the neck of the flask inside the fume hood. On continued heating the charred material changes in colour from brown to almost black and frothing

usually occurs. Remove the micro-burner, and heat very cautiously by just touching the bottom of the flask with the flame and quickly removing it. Overheating at this stage should be avoided otherwise the charred material may creep up into the neck of the flask. After a further few seconds when the frothing will have subsided replace the flame for vigorous boiling to continue. When the frothing ceases dense white fumes fill the flask, condensing on the sides and serving to wash down and digest any carbonised material adhering to the sides of the flask. The mixture becomes lighter in colour and clears in about 5–5·5 min. from commencement of boiling. The end-point is reached when the digest turns a bright greenish-blue. Prolonged heating will always cause a precipitate to form, although this can be removed by centrifuging after nesslerisation, without absorbing any colour. As soon as the digestion is complete the flask should be removed from the flame and cooled in running water. After cooling the digest darkens slightly in colour. Quantitative figures recorded in the appended table show that the slight carbonisation is not due to incomplete digestion of the protein.

Add about 30 ml. of water to the flask as soon as possible and warm gently over the micro-burner to dissolve the thick syrup. Transfer the extract to a 250 ml. flask, and wash out the digestion-flask with liberal quantities of water, transferring the washings to the 250 ml. flask. Dilute to the mark with distilled water and mix well. Measure 50 ml. into a 100 ml. graduated flask, and add 30 ml. of Nessler's reagent. Dilute to the mark with water and mix thoroughly.

Prepare the standard at the same time by adding to about 60 ml. of water in a 100 ml. flask 3 ml. of the standard ammonium sulphate. Finally 1 ml. of digestion mixture is added, and after mixing, 30 ml. of Nessler's reagent. Dilute to 100 ml. with water and again mix well.

Compare in the colorimeter setting the standard at about 25 mm.

Calculation:

$$\frac{\text{Standard}}{\text{Unknown}} \times 157 = \text{mg. N/100 ml. human milk.}$$

Since 1 mg. of nitrogen is equivalent to 6·38 mg. of milk protein, the above equation multiplied by $\frac{6{\cdot}38}{1000}$ gives the percentage of protein in human milk. For cow's milk multiply final result by two.

In order to prove the efficiency of the zinc hydroxide precipitant, the results obtained were checked by estimating the non-protein nitrogen, and by comparing with values for protein and non-protein nitrogen ascertained by using the tungstic acid precipitation in a form which is well known to have a wide application to biological fluids generally, particularly in regard to the estimation of the non-protein nitrogenous constituents.

The tungstic acid precipitation was carried out as follows:

To 5 ml. of a saturated solution of sodium sulphate in a centrifuge-tube add 1 ml. of human milk or 0·5 ml. of cow's milk. Add 2 ml. of 10 % sodium tungstate followed by 2 ml. of $\frac{2}{3}$ *N* sulphuric acid and shake vigorously. Immediately centrifuge at high speed (over 2000 r.p.m.) for 15–20 minutes, otherwise if the precipitate is allowed to flocculate before spinning it is impossible to obtain a clear supernatant fluid after centrifuging. Pour off the supernatant fluid. A little scum of precipitated fat may collect on the surface of the supernatant fluid, but this is of no consequence. Break up the precipitate by tapping the tube firmly with the finger, and then add 5 ml. of the saturated sodium sulphate *plus* 2 ml. of sodium tungstate and 2 ml. of the sulphuric acid. Mix by shaking vigorously and immediately centrifuge for 20 minutes. Carefully

pour off the supernatant fluid and transfer the precipitated protein to the silica flask as recommended in the standard technique. Proceed then as for the recorded method.

*Estimation of non-protein nitrogen.*

Pour off the first and second supernatant fluids obtained from the zinc hydroxide or tungstic acid precipitation into one of the silica flasks. Add 5 ml. of digestion reagent and digest in the usual way until the mixture is a bright greenish-blue and free from all carbon. Cool the flask in running water, and after dissolving the syrupy residue in about 10 ml. of water, transfer to a heat-resistant pyrex test-tube (22 × 200 mm.). Rinse out the flask with two 5 ml. portions of water adding these to the pyrex tube.

Liberate the ammonia by the addition of 20–25 ml. of 40 % sodium hydroxide. The aeration process follows that of the blood-urea method employed in this laboratory [Patterson, 1925], except that to prevent loss of ammonia during the addition of the alkali the tube containing the caustic soda is placed with its inlet tubes reversed, between the 5 % sulphuric acid tube used for removing the ammonia from the air, and the large boiling-tube containing the digestion extract, so that when the aeration is started the caustic soda is drawn over into the digested extract. The ammonia is drawn over into 5 ml. of $N/100$ sulphuric acid containing 3–4 drops of an indicator consisting of 100 ml. of 0·02 % alcoholic methyl red and 10 ml. of 0·1 % methylene blue.

Continue the aeration for half an hour, and after the first 5 minutes turn on the pump until 1 in. of foam is produced in the titration-tube. Back-titrate with $N/100$ sodium hydroxide.

Using the amounts specified in the methods, every 1 ml. of $N/100$ acid neutralised represents 0·00014 g. nitrogen for human milk, and 0·0028 g. for cow's milk. Table I illustrates the results of a few experiments on human and cow's milk.

Table I.

| Specimen No. | Direct digestion mg. protein per 100 ml. | Tungstic acid precipitant mg. protein per 100 ml. | Zinc hydroxide precipitant mg. protein per 100 ml. | Slight carbonisation of digest (see p. 1197). mg. protein per 100 ml. | Non-protein nitrogen estimated directly in supernatant fluid from protein precipitant mg. per 100 ml. | | N.P.N. calculated from difference between direct digestion and precipitation by tungstic acid and zinc hydroxide mg. per 100 ml. | | Urea (including preformed ammonia) mg. per 100 ml. | Free ammonia in terms of urea mg. per 100 ml. | Age of specimen before analysis hrs. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Tungstic acid | Zinc hydroxide | Tungstic acid | Zinc hydroxide | | | |
| Human milk: | | | | | | | | | | | |
| 1 | 1356 | 1017 | 1020 | — | 54 | 53 | 53 | 52 | 47 | 23 | 24 |
| 2 | 1130 | 714 | 700 | — | 63 | 63 | 65 | 67 | 43 | 16 | 48 |
| 3 | 1236 | 1012 | 1008 | 1003 | 33 | 39 | 35 | 36 | 25 | 0 | 2 |
| On 2 ml. milk | 1156 | — | — | — | — | — | — | — | — | — | — |
| Cow's milk: | | | | | | | | | | | |
| 1 | 2800 | 2260 | 2220 | — | 70 | 96 | 84 | 90 | 70 | 37 | 72 |
| 2 | (2702 | 2564 | 2526 | — | 28 | 29 | 25 | 27 | 22 | 0) | 2 |
| | (2732 | 2380 | 2414 | — | 42 | 38 | 55 | 49 | 39 | 17) | 48 |
| 3 | 2631 | 2362 | 2372 | 2353 | 36 | 44 | 42 | 40 | 30 | 0 | 2 |
| On 1 ml. milk | 2629 | — | — | — | — | — | — | — | — | — | — |

Experimental results show the close agreement between the zinc hydroxide and tungstic acid precipitations. The zinc process has the advantage of being less time-consuming and also less cumbersome. The marked increase with the

directly digested milk is due to the presence of the non-protein nitrogen, showing the inaccuracy of the older methods based on total nitrogen.

As already stated even prolonged heating of the digestion mixture does not prevent a very slight darkening developing after cooling of the digest. The results recorded in the column represented by "slight carbonisation of digest" were obtained by discontinuing heating just before the end-point of the digestion process, and this gave rise to greater carbonisation of the digest than that obtained by cooling after full digestion. Despite this fact the figures practically correspond with those obtained by the authentic digestion.

The actually determined non-protein nitrogen results are compared with the figures obtained by subtracting the total protein estimated by either tungstic acid or zinc hydroxide precipitation, from that found by the direct digestion of the milk and dividing by 6·38.

SUMMARY.

1. A quantitative method is described for the routine estimation of protein and non-protein nitrogen in human and cow's milk employing zinc hydroxide as protein precipitant.

2. By choice of selected proportions and sequence of reagents direct nesslerisation is possible, thus avoiding time-consuming methods such as distillation or aeration.

3. A true total protein is obtained and not a total nitrogen estimation.

The above investigation was carried out by kind permission of Dr J. Patterson, whose aid and advice greatly facilitated the progress of the work.

REFERENCES.

Denis and Minot (1919). *J. Biol. Chem.* **37**, 353.
Folin and Denis (1916). *J. Biol. Chem.* **26**, 473.
—— and Wu (1919). *J. Biol. Chem.* **38**, 81.
Patterson (1925). *Biochem. J.* **19**, 601.
Pyne (1932). *Biochem. J.* **26**, 1006.

# CLXIII. TYROSINASE IN MACROLEPIDOPTERA.

By CAECILIA ELISABETH MARY PUGH.

*From the Department of Physiology, The University, Manchester.*

(*Received June 15th, 1934.*)

## I. Distribution of tyrosinase.

The work of a number of investigators has shown the presence of tyrosinase in various species of Macrolepidoptera, but it is not clear from the literature whether the occurrence of tyrosinase is exceptional or general. A survey has therefore been made of some widely representative species, comprising four Rhopalocera (butterflies) and 25 Heterocera (moths). Since most tyrosinase is usually found in the larval form of an insect, this was the form chosen for the investigation. The tyrosinase is found in the body fluid.

### *Method.*

The larvae were chloroformed and dried on filter-paper. The skin was then snipped with scissors and the juice squeezed out by gentle massage. The juice was clear, except for a few parasitised larvae. It was added at once to 3 ml. phosphate buffer $p_H$ 6·5 by collection in a fine-pointed teat pipette which was washed out with the buffer solution. Of medium-sized larvae about six specimens were used; of large larvae, fewer were necessary; of small larvae, a greater number.

Of this preparation, 0·5 ml. was added to 2·5 ml. each of the following test solutions: (1) phosphate buffer $p_H$ 6·5 for comparison; (2) saturated solution of tyrosine in the same buffer; (3) 0·2 % *p*-cresol; (4) and (5) 0·2 % guaiacol; (6) 0·2 % catechol. To each was added 0·5 ml. water, except to (5), to which 0·5 ml. 0·05 % hydrogen peroxide was added instead. Coloration of tyrosine, *p*-cresol and catechol indicates the presence of tyrosinase; of guaiacol without hydrogen peroxide indicates laccase; of guaiacol with hydrogen peroxide indicates peroxidase.

*Species tested. Pieris brassicae, Pieris rapae, Melitaea aurinia, Augiades sylvanus, Chaerocampa elperor, Dicranura vinula, Orgyia antiqua, Arctia caia, Porthesia similis, Stilpnotia salicis, Lasiocampa quercus, Cosmotriche potatoria, Saturnia pavonia, Hipocrita jacobaeae, Triphaena pronuba, Barathra brassicae, Mamestra glauca, Diloba caeruleocephala, Miselia oxyacanthae, Xanthia lutea, Cucullia verbasci, Cheimatobia brumata, Abraxas grossulariata, Gonodontis bidentata, Crocallis elinguaria, Hybernia marginaria, Hybernia defoliaria, Phigalia pedaria.*

### *Results.*

There was no peroxidase in any of the juices tested and only a feeble laccase in some; but in every instance action on catechol appeared quickly, on *p*-cresol in a little longer time and on tyrosine after the lapse of either a few minutes or a few hours: tyrosinase was present in the juice of every one of the various kinds of larvae tested. (Exception: in one case the material available was insufficient to colour tyrosine itself.)

Several of the juices were placed on litmus paper and all tested were found to be alkaline. Some of the juices were noticed to darken very rapidly in air even in phosphate buffer $p_H$ 6·5.

*Conclusion.*

The presence of tyrosinase is quite general in the larvae of Macrolepidoptera, usually free from admixture with other similar oxidising enzymes.

*Note.* In sawfly larvae (*Trichiocampus viminalis*) the amount of tyrosinase was of a much smaller order, although the dead bodies darkened rapidly. The juice of 14 of these larvae overnight coloured only catechol, and *p*-cresol slightly. When 120 were used, action on catechol, *p*-cresol and tyrosine was obtained as rapidly as is usually obtained with about a dozen similar-sized larvae of the other types tested. Similar results were obtained with specimens from Euston and from Manchester.

## II. Tyrosinase in *Pieris* pupae.

*Pieris brassicae* (Large White) and *P. rapae* (Small White butterfly) are both double-brooded, and the black markings on the wings are much less distinct in the spring broods than in the summer broods.

The following experiments were carried out to determine whether there was any relationship between length of time in the pupal form, tyrosinase content of the pupae and markings of the emerged insect.

*Method.*

Pupae of both species were kept in a cool cellar for the winter. Similar pupae were incubated at 20°. From time to time tyrosinase activity was estimated as follows. The pupa was weighed and ground up in a small mortar with 1 ml. water. A further 2 ml. water were added, and the mixture was transferred to a centrifuge-tube. The mortar was washed out with a further 3 ml. water. The mixture was hand-centrifuged for one minute, the liquid was decanted and added to (1) 2 ml. phosphate buffer $p_H$ 6·5; (2) 2 ml. 0·2 % *p*-cresol solution in the same buffer; (3) 2 ml. saturated tyrosine solution in the same buffer. The time required for coloration to appear was noted. The usual precautions were taken to ensure freedom of the reagents from extraneous iron and *o*-dihydric phenols.

*Results.*

| | *P. rapae* | | | *P. brassicae* | | |
|---|---|---|---|---|---|---|
| | Weight g. | *p*-Cresol | Tyrosine | Weight g. | *p*-Cresol | Tyrosine |
| *Larvae slung up about to pupate.* | | | | | | |
| | 0·2702 | Nearly 1 min. | 10 mins. | 0·4392 | At once | 2 mins. |
| | 0·2228 | 1 min. | 10 mins. | 0·3168 | At once | 2 mins. |
| | 0·2118 | 2 mins. | 18 mins. | 0·2715 | Almost at once | 8 mins. |
| *Pupae 24–48 hours old.* | | | | | | |
| | 0·2288 | 6 hrs. | Overnight | 0·3336 | 3 mins. | 25 mins. |
| | 0·2300 | 3 hrs. | Overnight | 0·4034 | 20 mins. | 1⅓ hrs. |
| | 0·2040 | 3 hrs. | Overnight | | | |
| *Pupae 5 months old.* | | | | | | |
| Summer temp. | 0·1036 | 3½ hrs. | 5 hrs. | 0·3332 | 1½ hrs. | 1½ hrs. |
| Winter temp. | 0·1935 | 4 hrs. | 5½ hrs. | 0·3474 | 1 hr. | 4 hrs. |

Intermediate results were similar.

The tyrosinase content of individual pupae varied considerably, but it is evident that there was a very marked drop on pupation, and after that no actual disappearance of tyrosinase even after 5 months. Exposure of the pupae to chloroform vapour before extraction did not make the tyrosinase content any more regular (compare Graubard on *Drosophila* [1933]). The ratio of rate of coloration of *p*-cresol to that of tyrosine was extremely variable. On two occasions, after coloration of tyrosine had proceeded to some extent, the colour faded away, while *p*-cresol continued to colour. From this it would appear possible that the 5: 6-quinone of dihydroindole-2-carboxylic acid [Raper, 1927] may be able to function as a hydrogen acceptor and carrier. There was no regular difference between the tyrosinase contents of *P. rapae* pupae of different colours—green, grey or blackish.

After 2 months *P. rapae* at 20° began to emerge (mid-December). During the next few weeks altogether eight specimens emerged. In markings they were all intermediate between typical spring and summer broods. At this point an accidental interference with the temperature of the incubator resulted in the death of most of the *P. brassicae*. The survivors were used for estimations. The pupae in the cellar were brought to the laboratory in mid-April. During the next 3 weeks eight specimens of *P. rapae* emerged with typical spring markings, and two *P. brassicae*. The remainder of the pupae died.

### *Conclusion.*

The difference in intensity of markings of spring and summer broods of *P. rapae*, and probably *P. brassicae*, is not due to any difference in tyrosinase content, but to some other factor, depending on the temperature at which the pupae are kept. This conclusion is in agreement with the findings of Onslow [1916] that the markings are due to the action of tyrosinase on chromogen present in restricted areas of wing surface.

### REFERENCES.

Graubard (1933). *J. Genetics*, **27**, 199.
Onslow (1916). *Biochem. J.* **10**, 26.
Raper (1927). *Biochem. J.* **21**, 89.

# CLXIV. GOLD SOL REACTION IN IRRADIATED PROTEINS AND PROTEIN DERIVATIVES.

By MONA SPIEGEL-ADOLF.

*From the Department of Colloid Chemistry, D. J. McCarthy Foundation, Temple University, School of Medicine, Philadelphia, Pa.*

(*Received June 16th, 1934.*)

It has been previously shown [Spiegel-Adolf, 1934] that the specific protein tests become negative in diluted protein solutions after intensive irradiation with the light of a quartz mercury lamp. At the same time the reaction with colloidal gold becomes stronger and shows some new features. Evidence was supplied which proves that the substance giving this reaction was neither protein nor a typical protein derivative.

These studies have been continued on different lines chiefly with the purpose of obtaining more definite information as to the chemical nature of the substances formed during irradiation of proteins.

*Gelatin.* The investigations previously limited to water-soluble serum-proteins were extended to gelatin. According to Dakin [1920] 91·22 % of the amino-acid content of gelatin is known, so that it is one of the best characterised proteins. On the other hand, electrolyte-free gelatin [Spiegel-Adolf, 1927, 1], has no flocculating power towards colloidal gold, but only a marked protective power towards it. In this way all possibility that the precipitating effect of irradiated proteins is caused by traces of proteins which have escaped denaturation is excluded.

The gelatin used was made from a commercial brand (Coignet, Père et Fils & Comp., Gold Label). This brand was first purified by dialysis and electrodialysis. A sample of 2·1 % had an electric conductance of $1{\cdot}67.10^{-5}$ $ohm^{-1}$ (at 30° without water correction). 0·01 mg./ml. of this preparation protected 1 ml. of gold sol effectively against precipitation by 1·25 final normality of KCl. All the results were re-checked with different kinds of colloidal gold, made according to the method of Bredig or by reduction by formaldehyde. Both original samples and other samples purified by dialysis were used. The irradiations were made with the help of a quartz mercury lamp (Hanovia, 220 v. d.c.) at a distance of 15 cm. from the arc. In former investigations [Spiegel-Adolf, 1934] it had been ascertained that the protein derivatives which give positive gold sol reaction were heat-resistant. The container for the gelatin was a pyrex glass dish of 8 cm. diameter, the fluid layer being 1 cm. deep. This dish was covered by a plate of clear quartz. The gelatin was irradiated at a concentration of 0·04 % in order to get results comparable with those of the previous communication [Spiegel-Adolf, 1934], the greater transparency of gelatin in ultraviolet light permitting the work on a thicker layer.

At intervals of 30 minutes 3 ml. were taken and tested for their reaction with colloidal gold alone and in the presence of various KCl concentrations. Finally, with reference to the observations of Neuberg [1908], the reducing power of the solutions was tested.

The results are summarised in Table I.

Table I.

| Period of irradiation | Gold sol reaction | Protective power against KCl |
|---|---|---|
| 0 | 0 | $+1{\cdot}33\,N$ |
| 30 mins. | V | $+0{\cdot}2\,N$<br>$-0{\cdot}33\,N$ |
| 1 hr. | Bl | $+0{\cdot}2\,N$ |
| 1 hr. 30 mins. | Bl | $-0{\cdot}2\,N$<br>$+0{\cdot}1\,N$ |
| 2 hrs. | Bl | $+0{\cdot}1\,N$ |
| 2 hrs. 30 mins. | Bl | $0{\cdot}1\,N$ Rv<br>$0{\cdot}05\,N$ R |
| 3 hrs. | Bl | $0{\cdot}1\,N$ Rvvvv<br>$0{\cdot}05\,N$ Rvv |
| 4 hrs. | Bl | $0{\cdot}1\,N$ Bl<br>$0{\cdot}05\,N$ V<br>$0{\cdot}025\,N$ V<br>$0{\cdot}0125\,N$ R<br>$0{\cdot}0062\,N$ V |

R = cherry-red; V = violet; Bl = blue; suffixes v indicate shades of red with tinge of violet.

The findings show first that gelatin through irradiation becomes a precipitant for colloidal gold. It behaves in this respect like the other lyophilic proteins. The second change brought about by irradiation affects the protective power of the gelatin. Genuine gelatin does not need the presence of salts to exercise this power, as do serum- or egg-proteins, but under the influence of short-wave radiation the protective power of gelatin undergoes a change. As is shown in Table I gelatin flocculates colloidal gold after 1 hour of irradiation. Traces of unchanged gelatin if present are unable to prevent this flocculation. Yet no changes occur if a certain amount of salt is previously added to the gelatin after irradiation. With increasing irradiation time the amount of KCl necessary to prevent flocculation drops steadily, until it falls below the salt concentration which alone is able to precipitate colloidal gold. In a similar way Pauli and Singer [1932], studying the equilibria of colloidal Congo red, protein and salt, found that non-flocculating salt concentrations were able to protect the colloid against the precipitating effect of the protein.

In order to show that the behaviour of irradiated gelatin is not caused by contamination with the original gelatin, further experiments were started. The reactions towards colloidal gold of various mixtures of undenatured gelatin and of a gelatin that had been irradiated for 4 hours were studied. No salts were present (Table II).

Table II.

| Final conc. of non-irrad. gelatin % | Final conc. of irrad. gelatin % | Ratio of non-irrad. : irrad. gelatin | Gold sol reaction | |
|---|---|---|---|---|
| | | | Immediate | After 24 hrs. |
| 0·004 | 0·016 | 1/4 | 0 | Rv |
| 0·002 | 0·018 | 1/9 | 0 | R |
| 0·001 | 0·018 | 1/18 | 0 | Rvv |
| 0·0005 | 0·018 | 1/36 | 0 | Rvvv |
| 0·0002 | 0·01 | 1/50 | Rv | Rvvv |
| 0·0001 | 0·01 | 1/100 | Rv | V |

R = cherry-red; V = violet; suffixes v indicate shades of red with tinge of violet.

Only if the ratio of non-irradiated to irradiated material drops below 1/30 is a shifting of the red colour of the gold sol towards violet indicated. In order to get complete flocculation as observed with irradiated gelatin alone the content of original gelatin in the mixture ought not to exceed 1 %.

Though Zsigmondy [1920] has stated that the protective power of gelatin is increased by short heating, nevertheless, in order to refer the changes of gelatin to irradiation, influence of temperature had to be excluded. The temperature during irradiation fluctuated between 45 and 50°; a sample of gelatin of the same concentration and in a similarly covered dish was therefore kept in a thermostat for the same length of time (4 hours) at this temperature. After such an exposure gelatin did not show any marked difference from the original sample. It certainly did not flocculate colloidal gold, and it was able to protect the latter against the precipitating effect of high salt concentrations (1·25 *N* KCl) and of irradiated gelatin.

The changed behaviour of irradiated gelatin can therefore be considered as effect of denaturation through short-wave radiation. As in other proteins [Spiegel-Adolf, 1927, 2] denaturation by radiation is different from the changes caused by boiling.

But differences in the methods of irradiation influence largely the result. Although Neuberg [1908] refers to the reducing power of irradiated gelatin, similar tests made in our laboratory even after a hundredfold concentration of irradiated gelatin failed to give more than a dark greenish colour with Benedict's solution. Control experiments with dextrose and mixtures of dextrose and non-irradiated gelatin at various concentrations failed to give a similar reaction.

*Histamine.* Practically nothing is known about the exact chemical formulae of protein derivatives that show either marked protective or precipitating power for colloidal gold. As to the chemical changes brought about by irradiation of proteins there are pharmacological indications that small amounts of histamine are formed [Holtz, 1931]. On the other hand only lately Holtz [1933] has given chemical evidence of histamine formation on irradiation of histidine. Since no references [Feldberg and Schilf, 1930] as to the reaction of histamine with colloidal gold could be found, experiments were started in this direction.

Two different brands of histamine were used, histamine dichloride (W. A. Fritch, Inc., New York) and histamine diphosphate (Burroughs, Wellcome and Co., England). After neutralisation with NaOH the $c_H$ of the first solution was $1{\cdot}0 . 10^{-6}$, of the second $1{\cdot}41 . 10^{-6}$ at a concentration of 0·3 and 0·5% respectively.

In experiments re-checking former experiences [Adolf and Pauli, 1924] it was ascertained that HCl solutions causing visible changes in colloidal gold had a $c_H$ 20–30 times higher than those of the neutralised histamine solutions. In Table III are summarised results of a gold sol flocculation test by varying concentrations of histamine phosphate. Histamine chloride gave practically the same results when the concentration of the base was the same.

Table III.

| mg./ml. histamine | 0·000125 | 0·00025 | 0·000375 | 0·0005 | 0·00075 |
|---|---|---|---|---|---|
| Gold sol reaction | 0 | Rv | Rvv | V | V |

R = cherry red; V = violet; suffixes v indicate shades of red with tinge of violet.

By these figures it is shown that histamine is a very powerful flocculating agent for colloidal gold, visible changes being elicited by 0·00018 mg./ml. of the former. As histamine diphosphate contains only 36 % of free histamine the figures obtained are to be divided by about 3. The flocculating power of

histamine exceeds therefore not only the flocculating power of proteins but also that of irradiation products of proteins. In terms of normality it is 8 times stronger a precipitant for colloidal gold than $AlCl_3$ even though the latter is more acid [Adolf and Pauli, 1924]. When the gold sol reaction was made according to Lange's clinical liquor test with a neutralised 0·5 % histamine the diphosphate following results were obtained after 24 hours: 666666543211.

Experiments were now made in order to test the effectiveness of protecting material against gold sol flocculation by histamine.

Salt concentrations in a range of 0·1–0·04 *N* KCl were ineffective. Gelatin or combinations of serum-proteins and neutral salts when added previously to the colloidal gold prevented flocculation by histamine subsequently added. The correspondence of this behaviour to the one observed in irradiated proteins is rather obvious. It becomes still more striking when one considers that histamine, like the potent principle in irradiated proteins, shows heat-resistance when boiled in watery solutions, and also shows deterioration on standing, owing to growth of bacteria.

It is known that histamine formed through irradiation of histidine is destroyed by further irradiation. Experiments were started to investigate if the destruction of biological potency of histamine is accompanied by an analogous decline of the gold sol-flocculating power.

The biological potency of histamine was measured by its power to lower the blood pressure of a cat previously atropinised. 1 ml. containing 0·000005 mg. of histamine still gave a distinct reaction in a cat of 3 kg. This histamine concentration is far beyond the range giving positive gold sol reactions.

In order to obtain marked changes the neutralised histamine solution was brought to a concentration of 0·05 % and irradiated in the usual way for 4 hours. The reactions of irradiated histamine with colloidal gold showed that the flocculating power of the former was not completely destroyed, but that it had dropped to 1/100 of its original value. But when the irradiated histamine was examined as to its effect upon blood pressure, evidence could be given that its potency had shrunk to 0·00001 of its original value and came within the range of concentrations which yield positive gold sol reactions. These experiments show that histamine, though it is the most powerful gold sol precipitant known, owes these properties not exclusively to the molecular arrangement which causes its biological potency.

On the other hand, even if we suppose that the total histidine contained in gelatin—according to Kestner [1925] gelatin of mol. wt. 20,000 contains in 100 molecules 1 molecule of histidine—is changed by irradiation into a derivative of histamine having the full flocculating power of genuine histamine, the entire flocculating power of irradiated gelatin could still not be accounted for. The conclusion is therefore reached that gelatin and probably other proteins contain other constituents besides histidine which on irradiation are able to undergo such changes as to acquire gold sol-flocculating properties.

*Amino- and imino-acids.* A number of amino-acids are known to yield derivatives which are chemically analogous to histamine but more or less devoid of its biological potency. It was therefore supposed that those products might be formed by irradiation in the same way as histamine is formed from histidine. As a control histidine was included in a number of amino-acids which were subjected to irradiation.

Table IV contains a survey of the eight preparations examined, of the respective concentrations used and of the reactions with colloidal gold before and after irradiation.

Table IV.

| Name | $K_a$ and $K_b$ | mg./ml. | $c_H$ | Before irradiation | After 1 hour of irradiation | After 2 hours of irradiation |
|---|---|---|---|---|---|---|
| Histidine | $3{\cdot}9.10^{-10}$ | 0·15 | $2{\cdot}14.10^{-7}$ | 0 | | 0–72 hrs. 0 |
| | $1{\cdot}2.10^{-6}$ | 0·208 | | | 0 After 24 hrs. V | 0–24 hrs. V |
| | | | | | After 72 hrs. W | After 72 hrs. Bl |
| | | 0·625 | $1{\cdot}69.10^{-7}$ | 0–24 hrs. 0 | | |
| | | | | After 72 hrs. W | | |
| | | 3·5 | | After 3 weeks W | | |
| Arginine | $2.10^{-10}$ | 2·5 | $c_{OH}=1{\cdot}1.10^{-6}$ | | V | |
| | | | | | V–Blw | |
| | $2.10^{-6}$ | 2·5 | | | V–Blw | Blw |
| | | | | | Min. dos. > | Min. dos. = |
| | | | | | 0·1 mg./ml. | 0·17 mg./.ml |
| | | 5·0 | | After 72 hrs. 0 | | |
| Lysine | $3.10^{-11}$ | 0·6 | Neutral | After 72 hrs. 0 | 0–24 hrs. 0 | |
| | $2.10^{-5}$ | | | | After 48 hrs. V | |
| Proline | $2{\cdot}5.10^{-11}$ | 0·6 | $c_{OH}=1{\cdot}22.10^{-7}$ | 0–48 hrs. 0 | Bl | |
| | $1.10^{-12}$ | | | | Min. dos > | |
| | | | | | 0·06 | |
| | | | | | (alkaline) | |
| Glycine | $1{\cdot}8.10^{-10}$ | 1·0 | $K=2{\cdot}3.10^{-5}$ | 0–48 hrs. 0 | | 0–48 hrs. 0 |
| | $2{\cdot}6.10^{-12}$ | | | | | |
| Phenylalanine | $7{\cdot}5.10^{-10}$ | 0·25 | Neutral | 0 | | 0–24 hrs. 0 |
| | $4.10^{-13}$ | 0·5 | | 0 | | 0–24 hrs. 0 |
| Tyrosine | $7.10^{-11}$ | 0·16 | Neutral | 0–48 hrs. 0 | 0–42 hrs. 0 | |
| | $1{\cdot}7.10^{-12}$ | 0·23 | | 9–72 hrs. 0 | 0–42 hrs. 0 | |
| | | 0·46 | | | | 0–72 hrs. 0 |
| | | 0·6 | | | | 0–66 hrs. 0 |
| Tryptophan | $4{\cdot}1.10^{-10}$ | 0·075 | Neutral | 0–64 hrs. 0 | | |
| | $2{\cdot}2.10^{-12}$ | 0·15 | | | | 0–72 hrs. 0 |
| | | 0·3 | | 0–64 hrs. 0 | 0–64 hrs. 0 | |

0 = no reaction with colloidal gold. V = violet. Bl = blue. Blw = blue-white. W = white.

The choice of amino-acids was determined by the wish to include the most important constituents of gelatin. But in consideration of the previous work done on serum-albumin and pseudoglobulin tyrosine and tryptophan were also examined.

The concentrations of the solutions to be irradiated were chosen according to the different spectroscopic behaviour of the preparations which has been ascertained in every case.

As the reaction is supposed to influence the results of irradiation [Feldberg and Schilf, 1930], all solutions have been tested in this respect. If necessary the preparations were carefully neutralised and the results controlled. In all cases showing a reaction with colloidal gold $p_H$ determinations were made with the hydrogen electrode.

The same kind of solution of colloidal gold and the same method of irradiation were used as were used for gelatin. The optimum irradiation time (1–2 hours) was determined by experiments which showed that poorer results were obtained if the time was shorter ($\frac{1}{2}$ hour) or longer (3 hours).

Not one of the preparations flocculated colloidal gold before irradiation. Only the sample containing colloidal gold and histidine showed a change of colour after 72 hours at room temperature. A histidine solution which had been kept for 3 weeks in the ice-box flocculated colloidal gold at once. This behaviour may be explained by the bacterial formation of histamine [*cf.* Feldberg and Schilf, 1930].

Irradiation affects the preparations variously. While a 1 % solution of glycine remains colourless, the cyclic amino-acids, phenylalanine, tyrosine and tryptophan become dark brown. The other preparations turn more or less yellow. The dark brown colour interferes with the gold sol reaction. In order

to eliminate it the irradiated samples were acidified, whereupon a black-brown substance was precipitated. This substance could easily be redissolved in alkali, showing in this respect the same behaviour as melanic acid [Hoppe-Seyler and Thierfelder, 1924][1]. After some hours' standing, centrifuging or filtration a colourless fluid was separated. This was exactly neutralised.

Of the eight irradiated preparations four gave a positive gold sol reaction (histidine, arginine, lysine and proline), while the other preparations brought about no changes in the colloid. The type of reaction was the same as that described with irradiated proteins or with histamine.

The amount of irradiated histidine necessary to flocculate colloidal gold agreed approximately with the findings of Ellinger [1929], who proved that in the presence of oxygen 1/500 of the histidine is changed by irradiation into histamine. The flocculating powers of irradiated arginine and proline seem to come within a similar range. These facts suggest that the precipitating factor in irradiated histidine is for the greatest part histamine. The corresponding derivatives of arginine, lysine and proline would be putrescine, cadaverine and perhaps succinimide [Ciamician and Silber, 1913]. As has been shown above the great biological differences between those substances and histamine would not necessarily affect the gold sol-precipitating powers in the same degree.

Some suggestions will now be offered as explanations of the changed behaviour of these amino- and imino-acids towards colloidal gold after irradiation. As colloidal gold is a negative colloid, every increase of the basicity of a substance should increase its gold sol-flocculating power. In the case of proteins or amino-acids an increased positive charge can be conferred either by destruction of carboxyl or by increase of amino-groups. The three amino-acids, histidine, arginine and lysine, which give a positive reaction with colloidal gold after irradiation, are originally stronger bases than acids. The apparent dissociation constants given in Table IV are quoted from Kirk and Schmidt [1929]. The chemical difference between histamine and histidine consists in loss of the carboxyl group from the latter. Analogous changes can be supposed to take place in irradiated arginine and lysine. There is therefore some reason to suppose that irradiation enhances the positive charge of amino-acids by decarboxylation. Even when using an irradiation which destroyed more than 50 % of the histidine, Lieben and Getreuer [1933] failed to detect the formation of new amino-groups.

The same explanation can possibly be applied to the behaviour of irradiated proline. According to Bachér [1928] a solution of pyrrole when oxidised by irradiation becomes black. No such changes were observed in irradiated proline. Neither have Lieben and Getreuer [1933] been able to show a marked effect of irradiation on the cyclic ring of proline as measured by the amount of $NH_3$ set free. The fact that the solution becomes slightly alkaline on irradiation is accounted for by decarboxylation. The end-product may be identical with or closely related to succinimide.

The cyclic amino-acids, phenylalanine, tyrosine and tryptophan, behave in a different way. But it has been mentioned already that they show signs of deeper changes, *e.g.* formation of melanic substances. Results of spectroscopic measurements seem to point in the same direction.

The investigations of irradiated amino- and imino-acids offer evidence that other than histidine derivatives contribute to the gold sol-flocculating power of irradiated gelatin. According to Kestner [1925] 100 molecules of gelatin contain

[1] It may be interesting in this connection that Risak [1930] was able to trace melanin in the urine of a patient who had undergone an excessive exposure to sunshine with resultant dermatitis.

77 molecules of glycine, 1 of histidine, 10 of lysine and 10 of arginine. If compounds of amino-acids behave in irradiation like the latter alone, only a quarter of the constituents of gelatin are responsible for the gold sol-flocculating power of this protein after irradiation.

*Thymonucleic acid.* In order to ascertain whether other substances showing originally only protective power towards colloidal gold become flocculating after irradiation, a few experiments were made on thymonucleic acid and some pyrimidines. Zsigmondy [1920] states that nucleic acid has a gold number of 2·5., In two different preparations of thymonucleic acid carefully neutralised under control of hydrogen ion determinations, protective power for colloidal gold against the flocculating effect of salt could be detected. Neutralised thymonucleic acid alone did not apparently change the gold sol. In a previous communication [*cf.* Spiegel-Adolf, 1934], the importance of the dilution for the results of irradiation has been demonstrated. By spectrographical studies to be reported elsewhere [Spiegel-Adolf and Seibert, 1934] the concentrations penetrable by ultra-violet light were determined. Irradiation with the quartz mercury lamp was tried on thymonucleic acid samples of various concentrations (0·33–0·02–0·0033 %).

Even 6 hours' irradiation of the most concentrated sample did not change the protective power of thymonucleic acid. But in the more dilute samples it was sufficient not only to destroy any trace of protective power but also to produce a substance which could precipitate colloidal gold. (As the solutions became strongly acid on irradiation they had to be carefully neutralised.) These findings agree to a certain extent with references in the literature. Lieben and Getreuer [1933] working on 0·1 % solutions of thymonucleic acid found no changes following irradiation, while Heyroth and Loofbourow [1931] who used solutions of 0·0035 % and worked at 2 cm. distance from the mercury arc describe the disappearance of selective absorption after 2 hours' irradiation.

Though the participation of derivatives of other constituents of the nucleic acid cannot be entirely excluded, only a few irradiation experiments were made on some pyrimidines. Changes of the latter can be easily checked by spectrographical methods. Lieben and Getreuer [1933] have shown that on irradiation uracil is split with the formation of new amino-groups. A sample of uracil in neutralised solution after 2 hours' irradiation showed neither changes in the spectrum nor reaction with colloidal gold, but after 4 hours' further exposure the selective absorption disappeared and the solution precipitated the sol. Cytosine showed a similar behaviour on irradiation. A formation of new amino-groups, associated as it is with an increased basicity of the irradiated solutions as suggested by Lieben and Getreuer [1933], would explain these observations. The loss of protective power and the gain of precipitating power for colloidal gold after irradiation does not seem therefore a characteristic feature of protein colloids, alone, although the increase in positive charge may be reached by other ways.

*Spinal fluids.* In a previous paper [Spiegel-Adolf, 1934] some similarity was discussed between the behaviour of irradiated proteins and some pathological spinal fluids. The findings that irradiation and bacterial infection lead in some instances to the same result in producing substances which precipitate colloidal gold encouraged some further investigations along these lines.

In a preliminary series of experiments made with E. Spiegel ten pathological spinal fluids were tested according to their depressing effect on the blood pressure of previously atropinised cats, no equally sensitive test being known for the biologically non-active derivatives. Though some of the spinal fluids showed

the characteristic gold sol curve as described in general paralysis, no unquestionable proof of the presence of histamine could be obtained. However, it is a well-known fact that histamine is easily destroyed in the body.

The investigations will be continued in several directions.

## Summary.

1. Gelatin in the course of irradiation with a mercury arc lamp loses its protective power towards colloidal gold and gradually becomes a strong precipitant for the latter.

2. Histamine possesses a remarkable power of precipitating gold sol, as little as 0·00018 mg./ml. yielding a reaction. Irradiation does not destroy this property at the same rate as biological activity.

3. Arginine, histidine, lysine and proline flocculate colloidal gold only after irradiation, while glycine and the cyclic amino-acids do not do so even then.

4. Irradiation destroys the protective power of thymonucleic acid, which like cytosine and uracil becomes a gold sol precipitant.

The chemical and some biological aspects of these findings are discussed.

The author wishes to thank Dr Florence B. Seibert for kindly providing some of the preparations.

## REFERENCES.

Adolf and Pauli (1924). *Kolloid Z.* **34**, 29.
Bachér (1928). Handb. d. biolog. Arbeitsmet. her. v. Abderhalden, Abt. 1, Teil 2, H. 2, 1339.
Ciamician and Silber (1913). *Ber. deutsch. chem. Ges.* **46**, 558.
Dakin (1920). *J. Biol. Chem.* **44**, 499.
Ellinger (1929). *Arch. exp. Path. Pharm.* **136**, 129.
Feldberg and Schilf (1930). Histamin. (Springer, Berlin.)
Heyroth and Loofbourow (1931). *J. Amer. Chem. Soc.* **53**, 3441.
Holtz (1931). *Pflüger's Arch.* **226**, 559.
—— (1933). *Klin. Woch.* **12**, 1613.
Hoppe-Seyler and Thierfelder (1924). Handb. d. Physiol. u. Path. Chemischen Analyse. (Springer, Berlin.)
Kestner (1925). Chemie der Eiweisskoerper. 4th ed. (Vieweg, Braunschweig.)
Kirk and Schmidt (1929). *Univ. Calif. Publ. Physiol.* **7**, 57.
Lieben and Getreuer (1933). *Biochem. Z.* **259**, 1.
Neuberg (1908). *Biochem. Z.* **13**, 319.
Pauli and Singer (1932). *Biochem. Z.* **244**, 76.
Risak (1930). *Wien. klin. Woch.* **1**, 358.
Spiegel-Adolf (1927, 1). *Biochem. Z.* **180**, 395.
—— (1927, 2). *Biochem. Z.* **186**, 181.
—— (1934). *Biochem. J.* **28**, 372.
—— and Seibert. In press.
Zsigmondy (1920). Kolloidchemie, 3rd ed. (Spamer, Leipzig.)

# CLXV. THE IODINE CONTENT OF POTATOES.

By JESSE FRANCIS McCLENDON, EARL BARRETT and THOMAS CANNIFF.

*From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis.*

(*Received May 12th, 1934.*)

Potatoes were chosen by Remington as a staple food which is widely grown and might show an index of the iodine content of local foods of any locality, and we have therefore worked for some years on the technique of iodine determination in potatoes. This work is to some extent disappointing since we have found the iodine content of potatoes very low and very difficult to determine. The potato tops would be a better choice for iodine analysis since the iodine content is increased by the evaporation of water from the leaves and translocation from the roots, but they contain solanine which is toxic, and are therefore not used as food and are more difficult to obtain in distant localities. Towards the end of the season the tops are in various degrees of disintegration and are often entirely destroyed by potato beetles.

The potatoes were washed free of soil, sliced, dried in a current of air, ground in a coffee mill and dried again in an electric oven at 100°. Weighed samples of 50–200 g. of dry material were taken, and two methods were used for ashing. One was ashing in an electric furnace at 400° for about 36 hours. During the first part of this process whenever the sample showed any redness it was taken out and allowed to smoulder and then put back in the furnace when it had ceased smouldering.

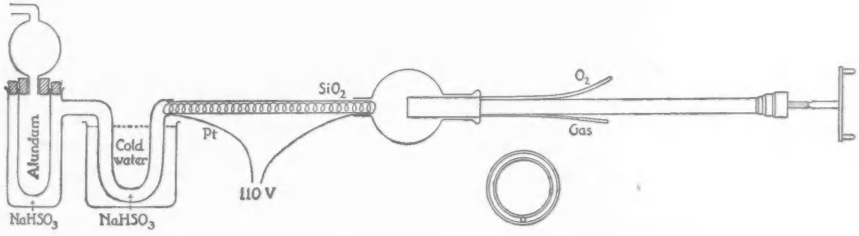



Fig. 1. Apparatus for burning dried tissue for iodine analysis. A detail of the opening of the screw-feed is shown below. The central circle is the space containing the dried tissue packed in a Visking sausage casing and around it is an annular opening from which oxygen issues to burn the tissue (the small circle below is an opening for a gas pilot flame).

The other method of ashing was in a combustion tube similar to the one used for seaweed [McClendon and Imai, 1933] except that the sample, instead of being pushed in gradually, was slowly introduced with a screw-feed (Fig. 1). The first part of the combustion tube is made of a one litre pyrex balloon flask with a hole in the bottom to receive an 8 mm. silica tube (containing an electrically heated

platinum wire) and the whole turned on its side until the silica tube is horizontal. The sample is dried, powdered, packed in a Visking sausage casing and placed in the screw-feed, the open end of which is surrounded by an annular opening through which oxygen is emitted to burn the sample. A very small pilot flame starts the combustion as soon as the screw is sufficiently advanced. The products of combustion pass over the heated platinum wire in the silica tube and are cooled in a pyrex U-tube and the iodine is absorbed by bubbling through two wash-bottles in series, only one of which is shown in the figure. Each wash-bottle contains an alundum capsule (RA 98) in $NaHSO_3$ solution, but sintered glass discs may be substituted for alundum to break up the bubbles and screen out the white smoke. This solution is evaporated with the washings of the apparatus and added to the ash.

The ash from either method of ashing is ground in a 200 ml. ball-mill with three portions (50 ml. each) of 85 % alcohol, which is evaporated, transferred to a boat, and heated in a simple combustion tube to which is connected a small wash-bottle containing $NaHSO_3$ solution and this solution together with the washings of the tube is added to the ash, which is rubbed up with a glass rod. To this extract is added a milligram of sodium azide, it is acidified with phosphoric acid and boiled down to 10 ml., transferred to a separating funnel and extracted twice with 1 ml. $CCl_4$. These extracts are set at 1 cm. in the micro-colorimeter (Bausch and Lomb) and compared with a standard containing 0·1 mg. $I_2$ per ml. $CCl_4$. Total iodine equals $\frac{x_1^2}{(x_1 - x_2)}$, where $x_1$ is the sum of 10 readings in cm. on standard balancing the first extract, and $x_2$ is the sum of 10 readings from the second extract. The result is the milligrams of iodine in the sample.

Fig. 2. The iodine in potatoes (γ/kg. dry matter) is shown in plain figures. The dotted line divides the eastern part of Minnesota lower in iodine and higher in goitre from the western part higher in iodine and lower in goitre. The figures in circles are the goitres too large to accommodate a military collar per 1000 men drafted during the World War. The numbers in squares are the percentage goitre in the schools.

It was found that there was a loss of iodine on burning in the electric furnace. Even after practising this technique for years there seemed no way to prevent this loss. An example is as follows: A composite sample of potatoes from western Minnesota was thoroughly mixed in the dried, ground condition by shaking in a large can and 50 g. were analysed in the combustion tube, showing an iodine content of 460 $\gamma$/kg. whereas in the electric furnace only 240 $\gamma$/kg. were recovered. Similar data for eastern Minnesota are 400 $\gamma$/kg. for closed and 310 $\gamma$/kg. for open ashing. The average of 55 samples of Minnesota potatoes burned in the electric furnace was 174 $\gamma$/kg. (see Fig. 2). The average of 28 samples from North Dakota burned in the electric furnace by another analyst was 170 $\gamma$/kg. (One sample from North Dakota burned in the tube gave only 140 $\gamma$/kg.) The main conclusion is that potatoes are very low in iodine content. If the wet weight is taken this is at once apparent. Owing to the fact that the water content of potatoes changes during shipment and storage we have made all determinations on the dry basis.

Although there are losses in the open ashing method at 400° we conclude from several years' experience that an analyst may develop a technique whereby his losses are comparable and by averaging a large number of determinations he may be able to tell whether the iodine content of potatoes of one region is higher or lower than that of another. This does not mean that these data may be compared with those of another analyst. The 55 samples of Minnesota potatoes analysed by the same analyst may be divided into those from the eastern and those from the western section of the State. The average value for the eastern section is 85 $\gamma$/kg. and the average for the western section 226 $\gamma$/kg. These figures may be compared with the simple goitre per thousand drafted men during the World War, which was 19 for this eastern section and 7·5 for this western section, indicating an inverse relation between the iodine in potatoes and the goitre in drafted men (Fig. 2).

REFERENCE.

McClendon and Imai (1933). *J. Biol. Chem.* **102**, 91.

# CLXVI. VITAMIN C IN THE ADRENAL GLANDS AND THE HYPOPHYSIS CEREBRI OF THE OX.

By BENEDICT WESTERGAARD.

*From the Institute of Hygiene, University of Copenhagen.*

(*Received May 18th, 1934.*)

SEVERAL attempts have been made lately to ascertain where the ascorbic acid is located in the tissue of the adrenal glands [Bourne, 1933, 1, 2; Giroud and Leblond, 1934].

The ability of ascorbic acid to reduce a solution of silver nitrate even in an acid medium has been used to demonstrate the occurrence of vitamin C in the tissues.

The experiments have been carried out in the following way:

(1) The fresh organs are frozen and cut in slices 2–3 mm. thick.

(2) Treatment with 0·4 % $AgNO_3$ ($p_H$ 4–4·5 by acetic acid or $HNO_3$) for 5–10 minutes.

(3) Washing in water.

(4) Treatment with 5 % $Na_2S_2O_3$ made acid by $NaHSO_3$ for 5–10 minutes.

(5) Washing in running water for 12 hours.

(6) Hardening in alcohol and preparation of paraffin sections.

(2), (3) and (4) are carried out in darkness; finally staining with eosin or haematoxylin-eosin.

Macroscopically the vitamin C-containing tissues are coloured black through an impregnation with free metallic silver in the places where the ascorbic acid has reduced the acid silver nitrate.

This impregnation begins immediately in the fresh tissue and is found only in the most superficial cell layers; this certainly depends upon a coagulation of the albuminous fraction of the tissue caused by the silver nitrate and upon a precipitation of AgCl. These substances are supposed to form a barrier for any further progress of the silver impregnation of the deeper cell layers.

## I. *The adrenal glands.*

The colouring begins rapidly in the cortex and is followed by a slower black impregnation of the medulla which finally turns completely black.

Microscopically (Fig. 1, Plate VII) the cortex shows in particular a staining of the cells of the zona fasciculata and reticularis.

The impregnation is seen as black granules of nearly equal size evenly dispersed in the cell cytoplasm but not appearing in the nucleus. In the sections stained with eosin the place of the nucleus is seen as an empty spot, free from granules.

Furthermore black granules are deposited in the intercellular tissue either arranged like strings of pearls along the interior of the lymph vessels and in the intercellular spaces or diffusely scattered over the tissue.

This arrangement will be discussed later.

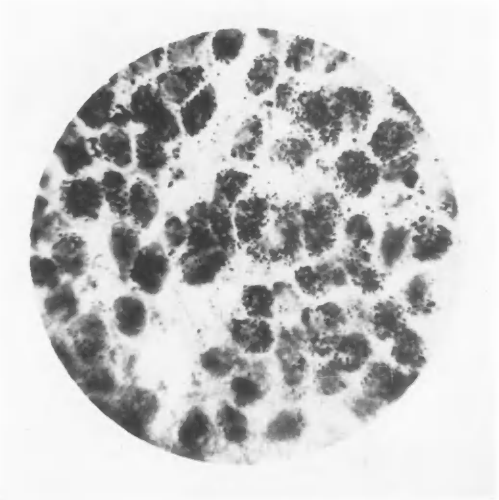


Fig. 1. Suprarenal bodies from ox stained with $AgNO_3$ and eosin.

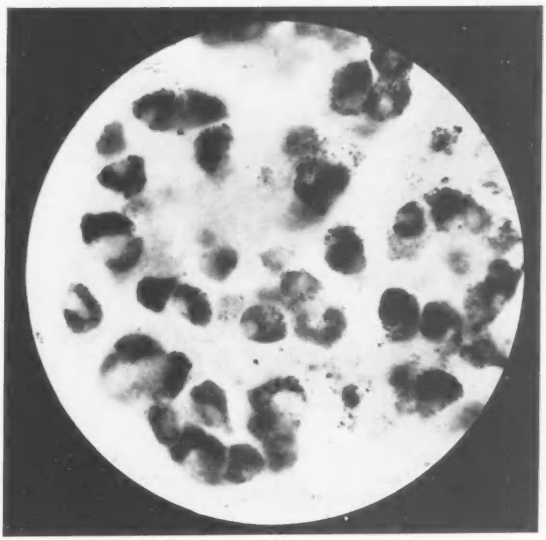


Fig. 2. Hypophysis from ox stained with $AgNO_3$ and eosin.

The medulla, which is built up by cells not so densely packed and more irregularly arranged than in the cortex, is completely covered with small silver granules staining both the cells and the intercellular tissue. The staining here looks very different from that of the cortex, the granules of the medulla being of a smaller size and covering the medullar tissue quite evenly so that this tissue looks as if it were covered with a grey carpet.

The sections stained with haematoxylin show that the silver impregnation is most pronounced in the cells in which the cytoplasm has a greater affinity to the acid than to the basic components of the stain solutions.

### II. *The hypophysis cerebri.*

Only the anterior lobe is stained.

Here the cells are not so densely packed as in the cortex of the adrenal gland and the staining extends to a greater depth from the surface.

Microscopically (Fig. 2, Plate VII) the cytoplasm of the cells is seen uniformly filled up with black granules of equal size. The granules, as with the suprarenal cortex, are not to be found in the nucleus.

In the sections stained with haematoxylin-eosin the silver staining is more pronounced in the cells with greater affinity to the acid than to the basic components of the stain solution.

As found in the adrenal cortex a number of black granules are also to be seen in the intercellular spaces, the lymph vessels and the connective tissue in the hypophysis cerebri.

It is very probable that these extracellular deposits of silver granules are also due to reduction of $AgNO_3$ by ascorbic acid which has passed the cell membranes either on its normal way from the cells to the tissues in other parts of the body and *vice versa* or through a postmortally injured cell membrane.

### REFERENCES.

Bourne (1933, 1). *Nature*, **131**, 874.
—— (1933, 2). *Nature*, **132**, 859.
Giroud and Leblond (1934). *Compt. Rend. Soc. Biol.* **115**, 841.

# CLXVII. THE ROLE OF 1:2:5:6-DIBENZANTHRACENE IN THE PRODUCTION OF FOWL TUMOURS.

By JAMES GOWANS CHALMERS.

*From the Research Department, The Glasgow Royal Cancer Hospital.*

(*Received May 19th, 1934.*)

The injection of 1:2:5:6-dibenzanthracene dissolved or suspended in fatty media into the breast muscle of fowls is followed, after a variable latent period, by the production of sarcoma in a significant number of birds [Burrows, 1933; Peacock, 1933]. Such tumours fulfil the accepted criteria of malignancy, in that they invade the normal tissues of the host and metastasise freely, giving rise to secondary tumours of similar histology. They can be transplanted in homologous series but so far have not been transmitted successfully by filtrates or desiccates.

The importance of determining the action of dibenzanthracene[1] in the production of these growths has been realised. Changes in rabbit serum-esterase values following intraperitoneal injection of a dibenzanthracene solution have been investigated [Green, 1934]. More recently it has been suggested that dibenzanthracene is altered in the body to form a water-soluble compound before it can produce biological effects [Boyland and Boyland, 1934].

The injection of a solution of dibenzanthracene in fat into the breast muscle of fowls might be followed by:

(1) Localisation of the dibenzanthracene in the muscle, or deposition in connective tissue or phagocytic cells.

(2) Active or passive removal from the site of injection.

(3) Chemical change.

The consideration of these possibilities involved an estimation of the quantity of dibenzanthracene present in the breast muscle of fowls at predetermined intervals after injection. No work appears to have been published on this aspect of the problem. A method for detecting dibenzanthracene in small quantities was necessary, since it was decided to inject amounts similar to those employed in producing experimental tumours. As a solution of 0·0005 % dibenzanthracene gave a well-defined absorption spectrum, the spectrographic method was used for its detection.

## Experimental.

The absorption spectrum of dibenzanthracene has been described by Clar [1929] who examined a benzene solution contained in an adjustable cell. In the present investigation, standard Hilger cells, which hold 5 mm. layers of liquid, were used for the spectrum analysis in conjunction with a Hilger Sector Photometer and Spectrograph (E, 3)[2]. A condensed iron spark was used as the source ultraviolet radiation. We are indebted to Dr Cook of the Cancer Hospital, London, for supplying a pure specimen of dibenzanthracene, which had been proved to be

[1] In every instance "dibenzanthracene" denotes "1:2:5:6-dibenzanthracene."

[2] The property of The British Empire Cancer Campaign.

carcinogenic by the London workers and which gave rise to fowl tumours in the experiments referred to previously. The dibenzanthracene absorption curve obtained corresponds closely to that given by Clar, and the differences observed are probably due to changes in technique. Fig. 1 shows the absorption curve over the range which is of special interest in this work (2600–3100 Å.).

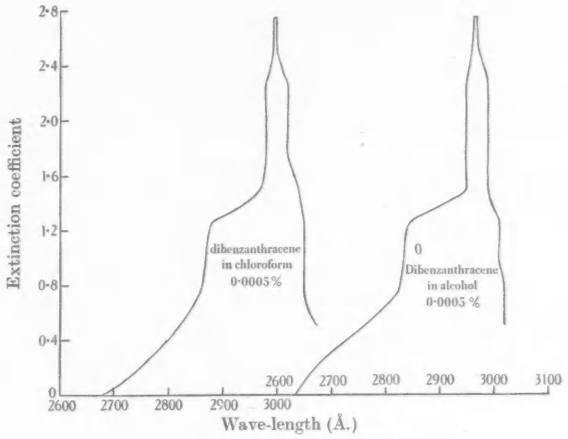



Fig. 1.

Dibenzanthracene (0·0005 %) in chloroform: extinction coefficient 2·8, maximum 2995–3000 Å.

Dibenzanthracene (0·0005 %) in alcohol: extinction coefficient 2·8, maximum 2965–2970 Å.

Extinction coefficient $= \frac{\log I_0/I}{d}$ where $I_0$ is the intensity of incident light, and $I$ the intensity of emergent light after passing through $d$ cm. of liquid.

The absorption curve of dibenzanthracene in chloroform when compared with the absorption curve given by Clar shows:

(1) A similar band with maximum at 3000 Å.

(2) An indication of a shallow band instead of a well-defined one at 2900 Å.

(3) No rapid increase in absorption below 2750 Å.

Thus, the chief difference occurs at wave-lengths less than 2750 Å.

It should be noted, however, that benzene, the solvent used by Clar, shows general absorption in this region.

*Preparation of extracts.*

In preparing a chloroform extract of an injected breast muscle, the same procedure was followed in all cases. The bird was killed and frozen to prevent loss of liquid fat, and the breast, together with the sternum, was removed; then the injected breast was excised, ground up with sand and extracted with chloroform in a Soxhlet apparatus for 6 hours.

*Preliminary experiments.*

Six fowls, injected with a solution of dibenzanthracene in egg-yolk fat or in lard with the object of producing tumours, which had died without showing any evidence of malignant change, were examined. The time from injection until

death varied from 18 months to 2 years. Where cysts or nodules had formed at the site of injection, these were examined separately, but otherwise the whole breast was extracted. None of the extracts, although concentrated to the point of general absorption in the ultraviolet, showed selective absorption. As this seemed to indicate that the dibenzanthracene had been removed from the site of injection or had changed into some other compound, it was decided to examine fowls killed shortly after injection.

*Series I. Dibenzanthracene in chicken fat.*

Four Plymouth Rock fowls (9 months old) were injected in the breast muscle with 5 ml. of a 0·10 % solution of dibenzanthracene in chicken fat.

| No. of fowl | Time from injection until death | Concentration of extract examined | Total dibenzanthracene in breast muscle at death (mg.) |
|---|---|---|---|
| 716 | Killed immediately | 1000 | 5·0 |
| 700 | 1 day | 1000 | 3·5* |
| 665 | 5 days | 1000 | 1·8* |
| 724 | 21 days | 1000>250 | Not detected |

* These approximate values are taken from the graph (Fig. 2), which shows the absorption spectra of breast muscle extracts from fowls 716, 700 and 665. A 0·5 % solution of chicken fat in chloroform did not exhibit any absorption in the ultraviolet.

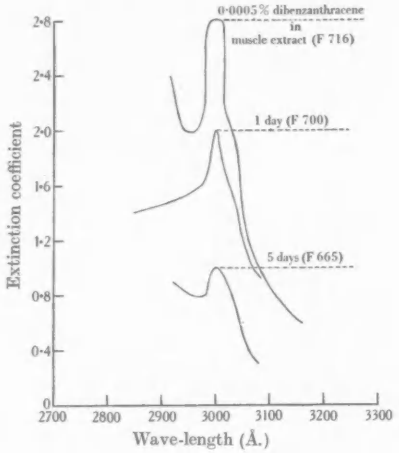



Fig. 2.

*Series II. Dibenzanthracene in egg-yolk fat.*

Seven Rhode Island Red chicks (6 weeks old) were injected in the breast muscle with 0·5 ml. of a 0·20 % solution of dibenzanthracene in egg-yolk fat.

The livers of chicks 1003, 1004 and 1007 were extracted with chloroform by a method similar to that adopted for the treatment of breast muscles. The extracts were examined spectrographically, but no dibenzanthracene could be detected.

The total chick excreta were collected during the course of the second series of experiments. A chloroform extract of these excreta, however, showed intense

| No. of chick | Time from injection until death | Concentration of extract examined | Total dibenzanthracene in breast muscle at death (mg.) |
|---|---|---|---|
| 1001 | Killed immediately | 200 | 1·0 |
| 1002 | 1 day | 200 | 0·6* |
| 1003 | 2 days | 200 | 0·5* |
| 1004 | 3 days | 200 | 0·4* |
| 1005 | 4 days | 200>50 | <0·1† |
| 1006 | 7 days | 200>50 | <0·1† |
| 1007 | 14 days | 200>50 | <0·1† |

* These approximate values are taken from the graph (Fig. 3), which shows the absorption spectra of breast muscle extracts from chicks 1001, 1002, 1003 and 1004; and the slight general absorption of 0·25 % egg-yolk fat in chloroform. The absorption spectrum of a 400 ml. extract from chick 1001 (*i.e.* a solution containing 0·5 mg. dibenzanthracene per 200 ml.) is inserted for purposes of comparison.

† To demonstrate that 0·1 mg. dibenzanthracene could be detected, 0·5 ml. egg-yolk fat solution containing 0·02 % dibenzanthracene was added to the breast muscle extract from chick 1005, which had not shown selective absorption. When re-examined with the spectrograph, the dibenzanthracene spectrum was observed.

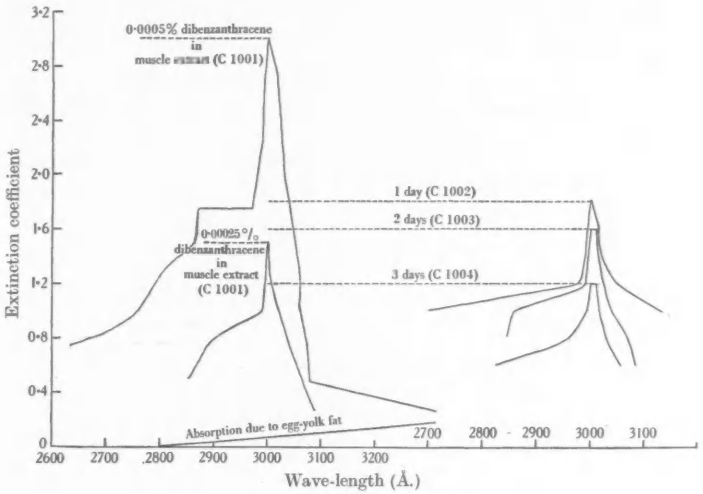



Fig. 3.

absorption in the ultraviolet, and it could not be tested until compounds which would mask the dibenzanthracene spectrum had been removed. Further work on this aspect of the problem is proceeding.

## Discussion.

The amount of dibenzanthracene present in the breast muscle of fowls injected with fatty solutions of this substance falls off rapidly from the time of injection, and after a few days decreases to less than one-tenth of the original quantity. This observation holds for all fowls examined, and it is worthy of note that the time taken for the dibenzanthracene to disappear is short compared with that required for clinical or histological evidence of a malignant change. It is evident that the bulk of the injected dibenzanthracene is transferred or under-

goes chemical change, though it is possible that minute quantities are phagocytosed and remain localised in this way.

No sign of dibenzanthracene has been found in the livers of any of the birds examined. The possibility of detecting dibenzanthracene in chick excreta is now under consideration. If dibenzanthracene is not found in the excreta the possibility of localisation in parts of the body other than the liver must be considered before any assumption of chemical change can be made.

## Summary.

1. The absorption spectrum of dibenzanthracene is described.

2. The quantity of dibenzanthracene present in the breast of the fowl, at short intervals after injection, has been determined and represented graphically to show that the dibenzanthracene rapidly disappears from the site of injection.

3. The possibility of the transference of this compound to the liver has been investigated, but its presence there has not been detected.

4. Examination of excreta to detect the possible elimination of dibenzanthracene as such has been attempted, but the tests applied have not proved satisfactory.

I have much pleasure in expressing my appreciation of Dr P. R. Peacock's advice throughout this investigation, and Mr S. Breslin's valuable assistance.

I am indebted to the British Empire Cancer Campaign for a personal grant.

## REFERENCES.

Boyland and Boyland (1934). *Biochem. J.* **28**, 244.
Burrows (1933). *Amer. J. Cancer*, **17**, 1.
Clar (1929). *Ber. deutsch. chem. Ges.* **62**, 353.
Green (1934). *Brit. J. Exp. Path.* **15**, 1.
Peacock (1933). *J. Path. Bact.* **36**, 142.

# CLXVIII. DILATOMETRIC STUDIES IN THE PROTEOCLASTIC DEGRADATION OF PROTEINS.

## II. PEPTIC HYDROLYSIS.

By MOTNAHALLI SREENIVASAYA and HEMMIGE BILLIGIRIRANGACHAR SREERANGACHAR.

*From the Department of Biochemistry, Indian Institute of Science, Bangalore, India.*

(*Received July 13th, 1934.*)

The present communication relates to a dilatometric study of the peptic digestion of caseinogen and gelatin, the experimental technique employed being similar to that described in one of our earlier contributions [1932]. Hammersten's "casein," Gold Label gelatin, and Pfanstiel's pepsin (1: 10,000) were the materials employed in the investigation. The reactions were conducted at $p_H$ 2·2, employing McIlvain's citrate buffer, and at a temperature of 30°. In each case two different concentrations of the protein have been employed.

### Results.

Figs. 1 and 2 represent graphically the course of peptic hydrolysis of caseinogen and gelatin, as followed by the dilatometer (Fig. 1) and the determination of amino-nitrogen (Fig. 2) by Van Slyke's method. It is clear from the graphs that the kinetics of peptic digestion can be followed in the dilatometer not only with greater ease and elegance but also with greater accuracy, since the fall in volume per millimol release of amino-nitrogen is very much greater than the corresponding value obtained in the case of tryptic hydrolysis.

The relation between the dilatometric depression and the corresponding increase in amino-nitrogen at different stages of the reaction, is represented in Fig. 3 and the dilatometric fall in ml. per millimol release of amino-nitrogen at different time intervals is given in Table I.

Table I. *Dilatometric depression (μl.) per millimol amino-nitrogen.*

| Time in mins. | 10 | 20 | 30 | 40 | 60 | 90 | 120 | 150 | 180 |
|---|---|---|---|---|---|---|---|---|---|
| Gelatin, 3·0 % | 12·9 | 12·5 | 12·0 | 11·6 | 12·9 | 12·6 | 12·8 | 13·4 | 12·9 |
| Gelatin, 6·0 % | 13·7 | 12·0 | 11·9 | 11·4 | 11·2 | 10·0 | 10·4 | 10·7 | 12·9 |
| Caseinogen, 1·46 % | 34·6 | 26·4 | 23·7 | 21·7 | 19·8 | 18·2 | 17·5 | 17·0 | 16·9 |
| Caseinogen, 2·84 % | 29·0 | 26·8 | 24·8 | 23·7 | 22·2 | 21·4 | 21·1 | 21·3 | 21·4 |

It will be seen from the table and the graph (Fig. 3) that in the case of caseinogen, there is no apparent proportionality between the depression and the release of amino-nitrogen, even if the abnormal changes characterising the initial stages of the reaction are not taken into account, thus confirming the observations of Rona and Fischgold [1933]. In the case of gelatin, however, a rough

proportionality between depression and amino-nitrogen can be perceived to exist. The anomalous behaviour of caseinogen is easily explained by the fact

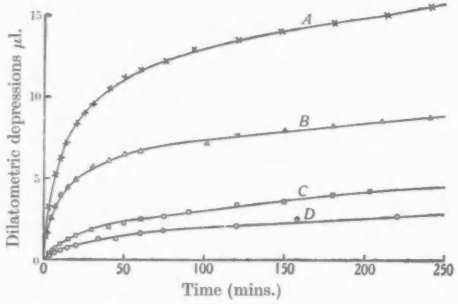



Fig. 1. *A* 2·8 % caseinogen; *B* 1·46 % caseinogen; *C* 6 % gelatin; *D* 3 % gelatin.

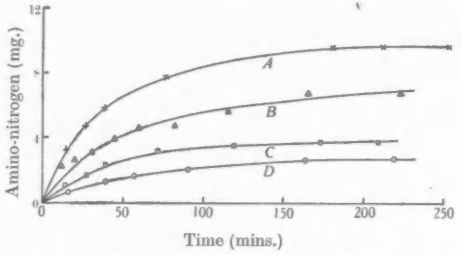



Fig. 2. *A* 2·8 % caseinogen; *B* 1·46 % caseinogen; *C* 6 % gelatin; *D* 3 % gelatin.

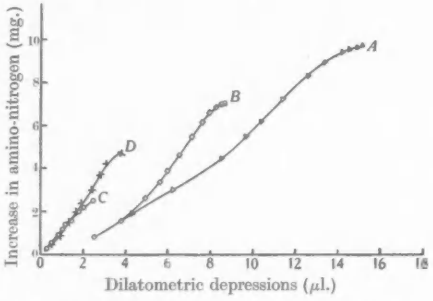



Fig. 3. *A* 2·84 % caseinogen; *B* 1·46 % caseinogen; *C* 3 % gelatin; *D* 6 % gelatin.

that during peptic digestion a precipitate of phosphopeptone appears and thus disturbs the homogeneity of the reaction mixture.

The average depression per millimol of amino-nitrogen (14 mg.) in the case of caseinogen is 22·9 and 12·0 for gelatin. Both these values are far higher than the

corresponding figures 10·8 and 8·7 respectively obtained during tryptic digestion [1934], thus pointing to an entirely different mode of attack of these proteins by pepsin. With a view to unravelling this mechanism of attack, a dilatometric study of the subsequent hydrolysis of the peptic hydrolysate by trypsin is being made.

SUMMARY.

1. The peptic degradation of caseinogen and gelatin has been followed in the dilatometer and also independently by a determination of the amino-nitrogen by Van Slyke's method.

2. In the case of caseinogen there is no linear relationship between the dilatometric depression and the corresponding amino-nitrogen released during the hydrolysis; in the case of gelatin, however, a rough proportionality exists as can be observed from Fig. 3 and Table I.

3. The average depression per millimol release of amino-nitrogen (14 mg.) in the case of caseinogen amounts to 22·9 while the value for gelatin is 12·0. These values, which are much higher than the corresponding values for tryptic hydrolysis, point to an entirely different mode of attack of these proteins in the case of pepsin.

REFERENCES.

Rona and Fischgold (1933). *Biochem. Z.* **261**, 366.
Sreenivasaya and Sreerangachar (1932). *J. Indian Inst. Sci.* **15** A, 17.
—— Sastri and Sreerangachar (1934). *Biochem. J.* **28**, 351.

# CLXIX. BERYLLIUM RICKETS.
## II. THE PREVENTION AND CURE OF BERYLLIUM RICKETS.

By HERBERT DAVENPORT KAY and DOROTHY IRENE SKILL.

*From the Department of Biochemistry, University of Toronto, and the National Institute for Research in Dairying, University of Reading.*

(*Received May 28th, 1934.*)

It was found in 1931 [Branion *et al.*, 1931] that severe rickets could be produced in rats by substituting beryllium carbonate for calcium carbonate in the Steenbock rachitogenic diet. Shortly afterwards the further observation was made that even on the normal stock diet [Bills *et al.*, 1931] of our rat colony with or without extra vitamin D in the form of irradiated ergosterol or cod-liver oil, very severe rickets (demonstrated by X-ray photographs, by the histological appearance of sections of the long bones, by bone ash and plasma-phosphate analyses) might be brought about in growing rats by the simple expedient of adding 0·5 % of beryllium carbonate to this normal diet [Guyatt *et al.*, 1933]. With smaller quantities of the carbonate down to about 0·1 % a milder rickets was produced, amenable in some degree to therapy with the usual antirachitic agents. The addition of an equivalent amount of beryllium phosphate to the Bills diet had no rachitogenic effect—the rats continued to grow normally.

It was shown further that in rats with beryllium rickets, both the inorganic phosphate content of the blood-plasma and the phosphoric ester content of the red blood cell were markedly reduced. This was also shown to be true in other types of experimental rickets [Kay, 1932].

In view of the unusual chemical property of beryllium in forming a phosphate which is relatively insoluble even in fairly acid solutions ($p_H$ 4 or less), *i.e.* at all acidities which are likely to occur in the intestine, it seemed not unlikely that the rachitogenic effect was bound up with this property. The beryllium going into the solution in the acid gastric juice would precipitate quantitatively in the intestine with any free phosphate found there, whether derived from the food or contained in secretions entering the intestine from various glands. The very insoluble precipitate would be excreted in the faeces and cause a constant drain on the animal's intake from the digested food, or re-intake from the intestinal secretions, of phosphate. It was further suggested [Kay and Guyatt, 1933] that the findings in beryllium rickets, taken together with those of other workers on experimental and human rickets, provided strong evidence for the view that experimental rickets, at least, is primarily a phosphate deficiency disease, and that the effect of vitamin D in physiological quantities is essentially to increase the uptake of phosphate through the gut wall, a view which, though not new, had not been supported hitherto by much weight of evidence. While it was obviously idle to endeavour to dissociate the metabolism of phosphorus from that of calcium, it was considered that variations from one individual to another in the functional efficiency of the gut wall in respect to phosphate uptake might

condition the rather irregular onset of rickets in different individuals of the same species on the same diet and might explain in some measure the marked difference in susceptibility to rickets from one species to another on the same diet.

The experiments recorded in this paper were designed to find out:

A. Whether on an otherwise normal diet, rendered rachitogenic by its beryllium carbonate content, the disease can be prevented by parenteral administration of phosphate.

B. Whether, and with what characteristic changes in the tissues, beryllium rickets can be cured by restoring the rachitic animal to a normal diet, *i.e.* by taking out the beryllium carbonate from the rachitogenic diet.

## Experimental.

Albino rats were used, the offspring of mothers living on the Bills stock diet or a slight modification of it. The rats after weaning were divided into groups of eight. They were placed on the experimental diets at 22–24 days of age and kept on them for 21 or 22 days. They were then killed by bleeding after mild anaesthesia, samples of tissues quickly removed and pooled for analysis, one complete hind leg was reserved for X-ray examination, and the bones of the other hind leg were used for determination of the percentage of ash in the dry, fat-free bone.

On a normal diet to which 0·5 % of basic beryllium carbonate had been added, very typical rickets developed rapidly and was fully established in 15–18 days, with all the characteristics previously described [Guyatt *et al.*, 1933].

### A. *The prevention of beryllium rickets by the parenteral injection of phosphate.*

One experiment may be described in some detail. Nine groups of 8 animals, with litters well shuffled, were used. To one of these groups (A) the normal diet was fed. To the other eight groups was fed the normal diet to which 0·5 % of beryllium carbonate had been added. Of these eight groups, one group (B) received no other treatment and was found with perfectly typical rickets at the end of 21 days. The next group (C) received in addition 1 ml. daily of 25 % neutral sodium $\beta$-glycerophosphate, given subcutaneously in two 0·5 ml. doses. Group D consisted of controls receiving instead of glycerophosphate, 1 ml. daily of a 10 % sodium chloride solution (of approximately the same osmotic pressure as the glycerophosphate), also in two 0·5 ml. doses. The other groups were given diminishing quantities of glycerophosphate or sodium chloride as shown in Table I. Sodium glycerophosphate was given in preference to sodium phosphate, since it is more soluble in water at $p_H$ 7·4 than the latter salt, so that a larger dose can be given in a smaller volume. It is, of course, readily hydrolysed in the body to sodium phosphate.

In Table II are shown values for plasma-inorganic phosphate, plasma- and kidney-phosphatase, phosphoric ester content of red cells and liver, and portion of ester-phosphorus of red cells readily hydrolysable by bone-phosphatase.

From Table II it will be observed that in all the animals with 0·5 % beryllium carbonate in the diet, irrespective of whether they received or did not receive parenteral glycerophosphate, the shortage of available P in their bodies, as demonstrated by the low inorganic P of their plasma and the low ester-P of the red cells and liver, was acute. Nevertheless two groups, C and E, had either normal or almost normal bone formation. It is evident that the parenterally injected and quantitatively far from adequate amounts of sodium glycerophosphate were made use of preferentially by the bone, the other tissues, which

Table I. *Effect of subcutaneous injection of sodium β-glycerophosphate in preventing "beryllium rickets."*

| Group | No. of animals in group | Av. gain in wt. over 21-day period g. | Diet and treatment | Results of X-ray examination after 21 or 22 days |
|---|---|---|---|---|
| A | 8 | 70 | Bills's stock diet | Normal. Excellent skeletal development |
| B | 8 | 24 | Bills's stock diet + 0·5 % beryllium carbonate | Typical severe rickets |
| C | 8 | 39 | Diet as for B, but 1 ml. of 25 % Na glycerophosphate at $p_H$ 7·4 given subcutaneously each day | 3 animals show very slight rachitic changes. Rest normal though less well grown than the A group |
| D | 8 | 26 | Diet as for B, but 1 ml. 10 % NaCl given subcutaneously each day | Rickets indistinguishable from B group |
| E | 8 | 32 | Diet as for B, but 1 ml. of 10 % Na glycerophosphate given subcutaneously each day | Less free from rachitic changes than group C, but all still nearly normal |
| F | 7 | 23 | Diet as for B, but 1 ml. 1 % NaCl given subcutaneously each day | Severe rickets |
| G | 8 | 24 | Diet as for B, but 1 ml. 5 % Na glycerophosphate given subcutaneously each day | All animals rachitic but much less affected than B or D groups |
| H | 8 | 31 | Diet as for B, but 1 ml. of 2·5 % Na glycerophosphate given subcutaneously each day | Typical rickets, but definitely less severe than in B, D, or I groups |
| I | 8 | 27 | Diet as for B, but 1 ml. 0·5 % Na glycerophosphate given subcutaneously each day | Rickets indistinguishable from B or D groups |

Table II.

| Group | Inorganic P in 100 ml. of blood (mg.) | Plasma-phos-phatase* (units per ml.) | Ester-P in 100 ml. of red blood cells: Total (mg. P) | Ester-P in 100 ml. of red blood cells: Easily hydrolysed (mg. P) | Ester-P in 100 g. liver (mg. P) | Kidney-phos-phatase* (units per 100 g.) |
|---|---|---|---|---|---|---|
| A. Normals | 7·7 | 0·96 | 80 | 18 | 92 | 28 |
| B. Severe rickets | 3·2 | 0·44 | 35 | 11 | 29 | 16 |
| C. Practically normal | 4·0 | 0·46 | 59 | 15 | 41 | 17 |
| D. Severe rickets | 3·2 | 0·39 | 54 | 10 | 30 | 15 |
| E. Almost normal | 3·4 | 0·45 | 62 | 14 | 31 | 17 |
| F. Severe rickets | 3·4 | 0·33 | 60 | 11 | 22 | 14 |
| G. Medium rickets | 3·6 | 0·52 | 64 | 11 | 26 | 17 |
| H. Rickets | 3·5 | 0·53 | 57 | 17 | 28 | 17 |
| I. Severe rickets | 3·7 | 0·59 | 52 | 17 | 31 | 18 |

Groups as in Table I.

* The arbitrary unit for phosphatase in plasma is not the same as the unit of phosphatase in kidney.

grew at a much slower rate than in group A, being still starved of phosphorus. This again would indicate that the production of rickets in the rat is more a question of keeping phosphate out of the body than of any damage to the intimate mechanism of bone deposition. The same remarkable fall in the phosphoric ester content of the red cells and liver previously noticed [Kay, 1932] in rickets

produced in rats by the Steenbock or McCollum rachitogenic diet is again shown. There is also a fall, the significance of which is at present unknown, in the kidney-phosphatase in all the animals receiving beryllium in their diet. It is perhaps to be mentioned here that the inorganic P content of the urine is very much diminished on the beryllium diet, though this has not yet been followed quantitatively.

B. *The rate of "cure" of beryllium rickets.*

The rate of "cure" of beryllium rickets after restoration of the rachitic animal to a normal diet (*i.e.* by taking $BeCO_3$ out again from the otherwise normal diet which had been rendered rachitogenic by the inclusion of 0·5 % $BeCO_3$) was followed by taking X-ray photographs, determining weight changes, the level of inorganic phosphate in plasma, and phosphoric esters in both red cells and liver at intervals after removing the $BeCO_3$ from the diet.

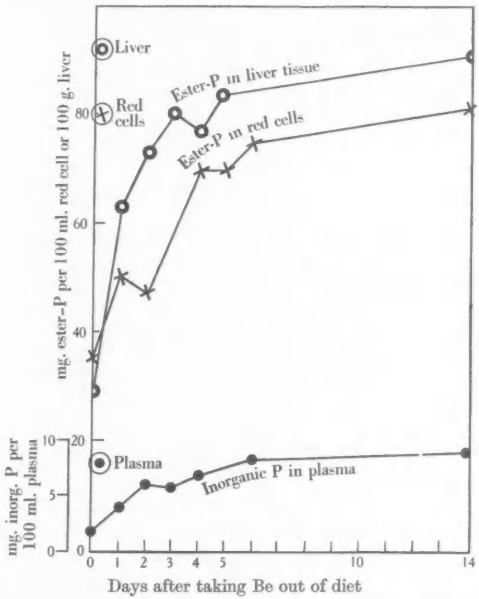



Fig. 1. The isolated points shown on left of graph, each surrounded by a circle, are average figures for the normal content of phosphoric esters in liver, P in red cells and of inorganic P in blood-plasma respectively, of normal animals of an age equal to that of the experimental animals on the day that Be was taken out of the diet of the latter groups.

An experiment of this kind with determinations at intervals of 0, 1, 2, 3, 4, 5 and 14 days after removing the rachitogenic $BeCO_3$ from the diet furnished the data shown graphically in Fig. 1. Other data from the same experiment are given in Table III.

Another experiment on the same lines gave essentially the same results.

It is quite clear that the rate of recovery from beryllium rickets is very rapid. Even after 2 days there are definite signs of healing, and at the end of 3 days, the

Table III. *Recovery from beryllium rickets.*

Except for group A, which received a normal diet from weaning, all the other groups at the age of 23 ± 2 days were given the diet containing 0·5 % beryllium carbonate (but otherwise normal) for another 22 days, then the beryllium carbonate was omitted, leaving the diet strictly normal, and groups of animals were then killed and examined at intervals.

| Diet | X-ray findings | Plasma-inorganic P mg. P per 100 ml. | Red cell ester-P mg. P per 100 ml. | Liver ester-P mg. P per 100 g. | Units phosphatase in kidney |
|---|---|---|---|---|---|
| Strictly normal stock diet from age of weaning | Normal | 7·7 | 80 | 92 | 28 |
| 0·5 % Be diet | Rickets + + + + | 3·2 | 35 | 29 | 16 |
| Be diet stopped 1 day | Rickets + + + + | 4·7 | 52 | 63 | 18 |
| Be diet stopped 2 days | Rickets + + + Definite signs of healing | 5·6 | 46 | 73 | 22 |
| Be diet stopped 3 days | Healing rickets | 5·3 | 70 | 80 | 28 |
| Be diet stopped 4 days | Healing rickets | 5·9 | 70 | 78 | 26 |
| Be diet stopped 5 days | Healing rickets | 7·3 | 75 | 84 | 28 |
| Be diet stopped 14 days | Healed rickets | 8·2 | 81 | 98 | 25 |

animal is, radiographically and chemically, well on the way to recovery. It would appear therefore that no very deep-seated, irreversible condition is brought about even in young and rapidly-growing rats by the beryllium feeding. This finding would further strengthen the view that the rachitogenic effect of the beryllium is not the result of possible cell damage due to active toxicity of the, at most, very minute quantity of Be that it is possible to imagine might be taken up from the food without being chemically detectable[1] in the tissues. (Against the toxicity hypothesis must also be ranged the complete inability of Be phosphate to produce rickets when added to the diet.) Nevertheless it must not be imagined that the animals are completely unscathed after the severe disorganisation which has taken place during the period of Be feeding. Even 4 weeks after replacing the rats on the normal stock diet, the X-ray photographs show, at some distance from the now quite normal metaphysis, definite callus-like bands in the long bones in the region which had been occupied by the (hypertrophied) zone of provisional calcification during the period on the Be diet.

*Is "beryllium rickets" due to an impurity in the beryllium carbonate?*

The suggestion was made to us that some of the effects of the Be diet might possibly be due to fluoride in the basic beryllium carbonate, which is said to contain, occasionally, a little $BeF_2$. Although this suggestion arose too late to test some of the earlier samples of beryllium carbonate used, two samples derived from the same firm from whom the earlier samples were obtained and a commercial sample from another source contained no detectable quantities of fluoride by the ordinary chemical tests. Moreover, Lamb *et al.* [1933] have recently shown that 0·043 % of sodium fluoride in the otherwise normal diet produces no serious effect in the normal rate of growth of the rat. If our results were even partly due to the toxic effects of fluorine, a large proportion (5 % or more) of the beryllium salt used must have been fluoride, $BeF_2$.[2] This was certainly not the case in the samples of beryllium carbonate tested. Moreover,

[1] No Be could be found in the ashed bones or ashed tissues (after removing the alimentary canal) of rats fed for three weeks on such a diet, using two qualitative methods [*vide* Guyatt *et al.*, 1933].

[2] Assuming the same toxicity for $\frac{1}{2}BeF_2$ as for NaF, this is probably a safe assumption. $\frac{1}{2}CaF_2$ is much less toxic than NaF (same authors).

when the carbonate was converted into phosphate, by the simple expedient of adding the necessary amount of dilute phosphoric acid and gently evaporating, all the rachitogenic effect disappeared. Also, as just described, even when beryllium is fed, the parenteral injection of sufficient Na glycerophosphate will prevent the rachitic symptoms. Taken together, the evidence appears to be convincing that fluoride poisoning is not concerned in the phenomena of "beryllium rickets."

SUMMARY.

1: Beryllium rickets which invariably results when young rats are given the normal stock diet to which 0·5 % of beryllium carbonate has been added, can be prevented if at the same time that the beryllium is being fed, a relatively small daily quantity of sodium glycerophosphate is administered parenterally. This adds further support to the view that beryllium rickets is mainly or possibly entirely due to defect of absorption of phosphate from the gut.

2. If animals with beryllium rickets are put on to a normal diet without beryllium, they recover very rapidly. One of the first changes to be observed is an increase in the phosphoric ester content of the erythrocytes and of the liver.

The authors wish to express their indebtedness to Prof. H. Wasteneys for his kindness in affording facilities for the completion of this work and for his generous help in other ways both before and after the senior author had left Toronto.

REFERENCES.

Branion, Guyatt and Kay (1931). *J. Biol. Chem.* **92**, xi.
Bills, Honeywell, Wirick and Nussmeier (1931). *J. Biol. Chem.* **90**, 619.
Guyatt, Kay and Branion (1933). *J. Nutrition*, **6**, 313.
Kay (1932). *J. Biol. Chem.* **99**, 85.
—— and Guyatt (1933). *Nature*, **131**, 468.
Lamb, Phillips, Hart and Bohstedt (1933). *Amer. J. Physiol.* **106**, 350.

# CLXX. NOTE ON THE PHOSPHORIC ESTER CONTENT OF THE RED CELLS AND LIVER, AND THE PHOSPHATASE OF THE KIDNEY IN EXPERIMENTAL OSTEOPOROSIS IN YOUNG RATS.

By DOROTHY IRENE SKILL and HERBERT DAVENPORT KAY.

*From the Department of Biochemistry, University of Toronto, and the National Institute for Research in Dairying, University of Reading.*

(*Received May 28th, 1934.*)

It has been shown [Kay, 1932] that in young rats given a rachitogenic diet (Steenbock 2965, McCollum 3143, or normal diet + 0·5 % beryllium carbonate) the phosphoric ester contents of the red blood cell and of the liver are markedly diminished. Recent experiments, one of which is detailed below, indicate that this effect is not confined to high Ca-low P diets, but may be shown equally on a low Ca-high P diet sufficiently severe to cause typical osteoporosis (demonstrated by X-ray photographs or histological findings). In the experiment shown in Table I several litters of the same age were divided into 5 groups of eight animals each and maintained under similar conditions on the different diets for 21 days before the determinations were made.

Table I.

Each figure is for the pooled blood or tissue from all the eight animals in each group.

| Diet | X-ray findings | Inorganic P in plasma mg. P per 100 ml. | Ester-P in 100 ml. of red cells mg. P | Ester-P in 100 g. of liver mg. P | Units phosphatase per 100 g. kidney |
|---|---|---|---|---|---|
| Normal stock | Normal | 6·4 | 79 | 89 | 28 |
| McCollum, 3143 | Rickets + + + + | 1·6 | 42 | 37 | 19 |
| Steenbock, 2965 | Rickets + + + | 2·7 | 42 | 37 | 22 |
| High P-low Ca | Osteoporosis | 4·9 | 44 | 47 | 15 |
| Steenbock, 2965 + C.L.O. | ? Very slight rickets in 1 animal; rest normal | 3·0 | 67 | 70 | 26 |

Table I shows that despite the excess of P in the osteoporosis-producing diet, the cessation or distortion of the normal process of bone growth was accompanied, as in true experimental rickets, by a marked lowering of both the ester-P content of the erythrocytes and liver, and the phosphatase activity of the kidney. In other experiments [Kay, 1932; Kay and Skill, 1934] it has been repeatedly observed that the amount of phosphatase per g. of kidney tissue in rats suffering from experimental rickets (in whatever manner induced), from experimental osteoporosis or from hypervitaminosis D is practically without exception lower than that found in normal litter-mate controls. The reason for this is not clear. It is evident, however, that another tissue must be added to the

list of those (bone, red blood cells, brain, liver) which have already been shown to become biochemically abnormal as the result of a gross imbalance between Ca and P in the diet.

SUMMARY.

In osteoporosis in rats caused by a low Ca-high P diet, despite the high phosphorus intake, the phosphoric ester content of unit volume of red blood cells or of unit weight of liver tissue is diminished. The phosphatase activity of the kidney is also reduced in experimental osteoporosis as in experimental rickets.

REFERENCES.

Kay (1932). *J. Biol. Chem.* **99**, 85.
Kay and Skill (1934). *Biochem. J.* **28**, 1222.

# CLXXI. THE DETERMINATION OF THE ISOELECTRIC AND ISOIONIC POINTS OF HAEMOGLOBIN FROM MEASUREMENTS OF MEMBRANE POTENTIALS.

By GILBERT SMITHSON ADAIR and MURIEL ELAINE ADAIR[1].

*From the Physiological and the Biochemical Laboratories and the Low Temperature Station, Cambridge.*

(*Received May 31st, 1934.*)

Hardy [1899] showed that the electrical charge of protein particles is negative in alkaline solutions and positive in acid solutions. The isoelectric point may be defined as the effective concentration or activity of hydrogen ions at which the protein is uncharged or possesses an equal number of positive and negative charges. Many of the properties of proteins, including solubility, osmotic pressure and cataphoresis, are correlated with the charge on protein ions and have been used in measurements of their isoelectric points, recently reviewed by Pauli and Valkó [1933].

The suggestion that the isoelectric point of a protein could be determined by measurements of membrane potentials has been made by Loeb [1924], Taylor [1924], Adair [1928] and Henriques [1929], but hitherto this has not been realised in practice. Loeb showed that acid solutions of gelatin are positive, whereas alkaline solutions are negative, but he was unable to obtain a definite value for the isoelectric point. The difficulties he encountered were partly due to the fact that gelatin is sparingly soluble at the isoelectric point and partly to the very low buffer value of the dilute acids and alkalis he used as dialysates. Taylor used haemoglobin and reported that the experiments were unsuccessful on account of precipitation of the protein at the isoelectric point. His solutions were acidified by the direct addition of acids. Adair [1928] obtained haemoglobin at the isoelectric point without precipitation by dialysis against buffer mixtures and stated that the isoelectric point in phosphate buffers is on the acid side of the isoionic point, defined by Sørensen *et al.* [1927] as the activity of hydrogen ions at which the protein neither gives off nor takes up hydrogen ions.

According to Henriques [1929] the "Donnan potential" of haemoglobin is zero at $p_H$ 5·5, a figure much more acid than the value $p_H$ 6·8 determined by the method of cataphoresis [Michaelis and Davidsohn, 1912]. Theoretical conclusions based on this work [Bolam, 1932] are of doubtful significance, as Henriques did not determine the equilibrium concentrations of phosphates and bases or the isoionic point of the protein, and the material used, a mixture of corpuscles and saponin, dialysed for 24 hours, was probably not strictly comparable with more highly purified preparations. There is a discrepancy between Henriques' experimental data and the results obtained by Adair [1928] and by Roche *et al.* [1932].

[1] Beit Memorial Research Fellow.

The sources of error in measurements of membrane potentials have been studied by Adair and Adair [1934], who devised a method adapted for systems with small potentials. The experiments described in the present communication show that it is possible to determine the isoelectric point of a protein by a similar method, and by correlating the measurements of membrane potentials with studies of the distributions of diffusible ions, made by chemical methods, the isoelectric point may be compared with the isoionic point of the protein.

### 1. *Preparation of CO-haemoglobin.*

Horse CO-haemoglobin was crystallised by a modification of the method of Ferry [1923], based on the fact that this protein is sparingly soluble at the isoelectric point. The haemoglobin of the sheep, like that of man and of the ox, is highly soluble at the isoelectric point and is therefore adapted for experiments which require a wide range of protein concentrations. In the preparation of proteins for measurements of isoelectric or isoionic points, a high degree of purification is advisable, but impurities must be removed under conditions which cause minimum changes in the protein molecule. In the present investigation, comparisons of preparations of sheep CO-haemoglobin, made by different methods, have been made, in order to reduce the risks of error due to impurities and denaturation products.

*Method 1.* This depends on the use of freshly purified ether and of sodium chloride to separate haemoglobin from the stroma-proteins and from lipins. Laboratory ether generally contains peroxides which cause methaemoglobin formation; the removal of peroxides can usually be effected by five extractions with $N/10$ sodium hydroxide, followed by four extractions with water. Even with purified ether, it is necessary to cool the mixture to 0° as soon as possible and to remove the ether by dialysis, as described below. The disadvantages attending the use of ether are compensated by a much more complete removal of stroma-proteins and of lipins than is accomplished by alternative methods based on the use of alumina cream [Walker and Williamson, 1920], centrifuging or certain processes of crystallisation.

For the preparation of sheep CO-haemoglobin, defibrinated blood was saturated with pure carbon monoxide and cooled for an hour at 0°. The plasma-proteins were removed by four centrifugings, 1·5 % NaCl being used to wash the corpuscles. A rubber-tipped glass rod was used to mix the corpuscles adherent to the bottom of the centrifuge-tube with the salt solution. The volume of the corpuscles was estimated, and for each 100 ml., 30 ml. of distilled water were used to wash the corpuscles into a flask, where laking was completed by the addition of 30 ml. of purified ether in the course of about 3 minutes' shaking. 13 g. of NaCl were then added, to shrink the swollen stromata, and the mixture was shaken until the salt had dissolved. After centrifuging (for about 10 minutes at 3000 r.p.m.) the stromata rise to the top in the form of a solid cake, and the clear solution of haemoglobin can be removed by means of a pipette. If contamination by stroma proteins occurs, it may be removed by filtration through cotton wool.

The haemoglobin solution was then placed in collodion membranes and dialysed against distilled water at 0°, the water being changed about 6 times at intervals of one hour on the first day and at three hour intervals on subsequent days. Overnight, the membranes were left in a volume of water at least 10 times as great as the volume of the membrane. In order to concentrate the solutions, the membranes were connected with reservoirs of air or carbon monoxide, under a positive pressure of about 700 mm. Hg. After dialysis against distilled water

for 3 days, the protein was dialysed against buffer solutions, as described in the following section.

*Method 2.* The corpuscles were laked with an equal volume of distilled water, without using ether, and 1 % NaCl was then added. A part of the stroma-protein could be removed by centrifuging, and further purification could be effected by pressure dialysis against distilled water, when a gelatinous precipitate formed on the membrane. It was generally necessary to change the membrane several times to ensure efficient dialysis and approximately complete removal of stromata.

*Method 3.* The solution freed from stromata and from ether and salt by method 1 was crystallised by the addition of ammonium sulphate at 0°. A 22 % solution of CO-haemoglobin was mixed with an equal volume of $M/15$ $Na_2HPO_4$, since a slightly alkaline reaction inhibits methaemoglobin formation; conditions favourable for crystallisation were then determined by trials with samples containing from 1·02 to 1·2 ml. of saturated ammonium sulphate per ml. of the diluted protein solution. Thin layers of the mixture exposed to the air usually crystallise within an hour or two, and these crystals can be used to seed the samples. The stock solution was then crystallised in a bottle containing carbon monoxide, with the addition of the determined amount of ammonium sulphate and about 5 ml. of previously crystallised solution. The mixture was shaken at intervals, and if necessary, after crystallisation had started, the yield of crystals was increased by the addition of more ammonium sulphate. The crystals could be separated from the mother-liquor by filtration through a Büchner funnel; they were then washed with a mixture of 110 ml. saturated ammonium sulphate and 100 ml. $M/15$ $Na_2HPO_4$ or $(NH_4)_2HPO_4$, saturated with carbon monoxide. The crystals were then dissolved in the minimum volume of water and dialysed. All the above operations were carried out at 0°. The yield was about 60 % of the total haemoglobin. Recrystallisation was brought about by the same procedure.

*Method 4.* The mother-liquor from the crystals prepared by method 3 was placed in large membranes and concentrated by dialysis against distilled water under a pressure of about 600 mm. Hg.

### 2. *Experimental methods for the determination of the isoelectric point from measurements of membrane potential.*

The apparatus for the measurement of membrane potentials, including collodion membranes and saturated calomel KCl electrodes, has been described in a previous paper [Adair and Adair, 1934]. In determinations of the isoelectric point, a series of membranes was prepared, filled with protein solution and placed in pyrex tubes or beakers containing a series of buffer solutions at different $p_H$ values, exemplified by the lists of solutions in Tables IV and V. The solutions and electrodes were kept in a room at $0° \pm 0·2$. The increase in the stability of the proteins and the inhibition of bacterial action at low temperatures made it possible to continue equilibration for 10 days or more, with repeated changes of buffer mixtures, and to make rigorous tests of equilibrium. If the membranes were fitted to manometers, osmotic equilibrium was determined as described by Adair [1925, 2]; if the membranes were stoppered with rubber bungs, the membrane potentials were determined at intervals until constant readings were obtained. Large volumes of dialysate were required for concentrated protein solutions equilibrated with dilute buffers.

By dialysis with repeated changes of buffer mixtures, it is possible to make a series of experiments with varying $p_H$ and constant phosphate concentration, or at a constant ionic strength. In the experiments of Loeb [1924], of Taylor [1924]

and of Henriques [1929], which were carried out at room temperature, a single sample of dialysate was used, in order to reduce the time of equilibration, but the equilibrium concentrations of the salts in the dialysate in such cases may differ quite markedly from the initial concentrations, and the relationship between the membrane potentials and the $p_H$ values is more difficult to interpret, if the salt concentrations are independent variables. Comparative studies of the membrane potentials of different protein preparations in equilibrium with the same dialysate are moreover facilitated by methods in which the dialysate is renewed repeatedly. This procedure has the additional advantage that traces of acidic and basic impurities are removed more rapidly by dialysis with solutions of electrolytes than with pure water.

After equilibrium has been established, two procedures are available for the determination of the isoelectric point

1. The $p_H$ values of the protein solutions and the dialysates are determined by the Clark hydrogen electrode or by other methods. In the more acid solutions, the $p_H$ of the protein solution exceeds that of the dialysate; in the more alkaline solutions, the $p_H$ of the protein is lower, as shown in Table XV, and it is possible to determine by interpolation the isoelectric point I, where both $p_H$ values are equal, according to the definition suggested by Adair [1928].

2. The membrane potential $E$ and the $p_H$ of the dialysate are determined for each of the systems, and the point where $E=0$ is found by interpolation, as shown in Fig. 1. If the protein concentrations are not identical, a correction can be applied, based on the observation that the ratio $E/C_v$ is approximately constant, when $E$ is small [Adair and Adair, 1934].

Theoretically, the two methods should give the same results, since the membrane potentials and the $p_H$ values of the protein solution and the dialysate, symbolised $p_{H'}$ and $p_{H''}$, are correlated by a thermodynamical formula due to Donnan and Allmand [1914], equivalent to formula 1 below, in the case of measurements at 0°, as stated by Adair and Adair [1934]:

$$E=54{\cdot}16\ (p_{H'}-p_{H''}) \qquad \ldots\ldots(1),$$

$E$ = membrane potential in millivolts at 0°.

In this equation and in all the formulae and tables given below where it is necessary to distinguish between the protein solution and the dialysate, the same notation is used. The symbols with two accents refer to the dialysate. Method 2 is preferable in the case of haemoglobin solutions on account of the difficulties of hydrogen electrode measurements in the protein solutions. Both methods are based on the assumption that a saturated solution of potassium chloride annuls the liquid junction potentials, and therefore the results may be regarded as conventional rather than as absolute values.

In special cases where the volume of the solution available is very small, approximate determinations may be made by a third method, in which the protein is equilibrated with a dialysate at a reaction close to the isoelectric point. The hydrogen ion concentration of the protein solution is estimated by formula 1, and the apparent valence $n_p$ by formula 9, and if $n_p$ be not equal to zero, a correction is applied, based on the observations recorded in Section 9, which show that over a limited range of $p_H$, the rate of change of valence with the $p_H$ is approximately constant.

The membrane potentials plotted against $p_H$, shown in Figs. 1 and 2, lie on smooth curves, and the point of intersection where $E=0$ can be determined with an accuracy which compares not unfavourably with alternative methods for the estimation of the isoelectric point. The degree of accuracy obtainable is increased

by the use of concentrated protein solutions, although the gain is not directly proportional to the protein concentration, since certain errors, including those due to liquid junction potentials, tend to increase with the protein concentration. In buffered solutions where the ionic strength is low (0·01–0·02), but not so low that traces of carbon dioxide from the air and alkalis from the glass vessels cause significant changes in the $p_H$ of the dialysate, it seems possible to estimate the isoelectric point of a given preparation with an accuracy of 0·01 $p_H$ unit. If the ionic strength of the buffer be 0·1 or more, the error may be 0·05 $p_H$ unit or even greater, as the potentials are small in concentrated salt solutions.

Measurements of isoelectric point are of little value without precise specification of the experimental conditions, including the composition of the solutions and the standardisation of the electrometric measurements. All the $p_H$ values of the dialysates, given below, were measured with a Clark hydrogen electrode, rocked for 1 hour at 0°, and a saturated calomel electrode. In order to obtain measurements on buffer solutions at 0° comparable with figures for higher temperatures, standardised as suggested by Clark [1928], the calomel electrode was standardised on the conventional assumptions that the $p_H$ of a solution of 0·01 *N* HCl *plus* 0·09 *N* KCl is 2·04, and that a saturated solution of KCl annuls the liquid junction potentials.

The protein concentration has been determined by the method of Kjeldahl, supplemented by measurements of densities and of refractions, as described by Adair and Robinson [1930, 1] and by Roche *et al.* [1932]. In order to calculate the percentage concentration of the protein, symbolised $C$, in g. dry protein per 100 ml. solution, the nitrogen content of sheep CO-haemoglobin was determined as $16{\cdot}75 \pm 0{\cdot}05$ % for crystallised and for uncrystallised protein. The percentage concentration $C$ is directly proportional to the difference between the densities of the protein solution and the dialysate, symbolised $D'$ and $D''$ in the formula $D' - D'' = k_d C$, where $k_d$ is an empirical constant, equal to 0·00257 at 0° and 0·00251 at 18°, in the case of solutions of sheep haemoglobin in equilibrium with dilute phosphate buffers. The refractive indices of the protein solution and of the dialysate, symbolised $R'$ and $R''$, are correlated with $C$ by the empirical formula $R' - R'' = \alpha' C$, where $\alpha'$ is equal to $0{\cdot}001945 \pm 0{\cdot}000005$.

The carbon monoxide capacity of the solutions of haemoglobin after equilibration was determined by the method of Van Slyke and Hiller [1928]. Estimations of diffusible substances present in the protein solutions and dialysates, namely phosphates, chlorides, ammonia and bicarbonates were made by methods referred to by Adair and Adair [1934]. Sodium was determined by electrodialysis [Adair and Keys, 1934]. The estimations of small amounts of bases, including ammonium and sodium, in the presence of large amounts of protein are subject to appreciable errors. Determinations of the ammonia content of the protein solution by nesslerisation gave in some cases higher values than those obtained by the micro-Kjeldahl method or by electrodialysis, some of which are included in Table VIII.

The results of analyses are usually expressed in g. mols. per litre of solution. The corrected concentrations, expressed in g. mols. per litre of solvent, may be calculated by formula 2, as described by Adair and Adair [1934]:

$$m_p = c_p \times 100/(100 - V_1 C);\ [NH_4]_c' = [NH_4]' \times 100/(100 - V_1 C) \quad \ldots\ldots(2),$$

$m_p$ = corrected concentration of the protein, of molecular weight 67,000; $c_p$ = protein concentration in g. mols. per litre of solution; $(100 - V_1 C)$ = volume of solvent in ml. per 100 ml. of protein solution; $C$ = g. dry protein per 100 ml.; $V_1$ = effective volume per g. dry protein, approximately 0·965 ml. The difference

between this figure and the specific volume of dry protein, 0·75 ml., is attributed to water combined with the protein. $[NH_4]_c'$ = corrected concentration of ammonium ions; $[NH_4]'$ = concentration of ammonium ions in g. mols. per litre of protein solution. In the dialysates, which are free from protein, the corrected concentration is the same as the observed concentration, $[NH_4]''$.

In addition to the measurements in which chemical analyses of protein solutions and dialysates were made for the purpose of comparing isoelectric and isoionic points, a number of determinations of isoelectric point were made by a simpler procedure, in which the measurements made included the membrane potentials, the refractive indices of the protein solutions and dialysates and the $p_H$ of the dialysates. If the protein solutions have been equilibrated with successive changes of dialysate, and the refractive indices and $p_H$ values of the final dialysate agree with those of the standard buffer mixture, it may be assumed that the composition of the dialysate is the same as that of the standard solution.

### 3. *The determination of the isoionic point of haemoglobin.*

Sørensen *et al.* [1927] have given a detailed description of the determination of the isoionic point of a protein in a form applicable to solutions of all protein concentrations. If, however, concentrated solutions are available, the estimation of the isoionic point can be made by simpler methods, since calculations given below show that there is little difference between the isoionic point and the hydrogen ion concentration of a concentrated solution of the protein in pure water, or in a solution containing equivalent amounts of crystalloid acids and bases.

Theoretically, such a solution is neither isoionic nor isoelectric, because the addition of a pure protein such as egg-albumin to water increases the acidity, so that the protein may lose hydrogen ions. In the case of egg-albumin, where the $p_H$ of pure solutions is approximately 4·8, the concentration of hydrogen ions given off is of the order of $2 \times 10^{-5}$ equivalent per litre, or 0·01 equivalent per mol. of protein, if the protein concentration be 0·002 *M*. The titration curves of Sørensen *et al.* [1927] show that egg-albumin is a protein with a relatively high buffer value, since one molecule (34,500 g.) gives off more than 8 hydrogen ions, if the $p_H$ of the solution be increased by 1 unit by the addition of alkali.

The loss of 0·01 equivalent of hydrogen ions per mol. corresponds to an increase of 0·0012 unit in the $p_H$, and therefore the $p_H$ of the pure solution may exceed the isoionic point by 0·0012 unit, a correction which can be disregarded in comparison with the experimental errors. At low protein concentrations, where the differences between the isoionic point and the $p_H$ value of the pure protein solution are more important, the correction cannot be estimated in the simple manner referred to above, and the determination must be made by the method of Sørensen.

The determinations of the isoionic point of haemoglobin made by previous workers have been obtained at various temperatures; the values may be reduced to 0°, using the temperature coefficient of 0·018 $p_H$ unit per degree [Adair *et al.*, 1929; Stadie and Martin, 1924]. The titration curves of Hastings, Van Slyke *et al.* [1924] show that the reduced haemoglobin of the horse has an isoionic point of 6·81 at 38° (7·495 at 0°), and the values for oxyhaemoglobin are 6·70 at 38° (7·385 at 0°), with a maximum deviation of 0·07 $p_H$ unit. Terroux [1931], who used the glass electrode, obtained the figure 6·79 at 22° (7·176 at 0°) for sheep haemoglobin; Errera and Hirschberg [1933] give 6·55 at 18–20° (6·87–6·91 at 0°) for horse haemoglobin. The results of different workers are thus not in agreement. The

data of Hastings, Van Slyke *et al.* [1924] are of special value as they refer to analysed solutions of known $CO_2$ and oxygen content.

In the present investigation, three methods have been used for the determination of the isoionic point. By method A, a number of measurements were made by a procedure similar to that of Sørensen *et al.* [1927] and of Van Slyke *et al.* [1922]. A concentrated solution of sheep CO-haemoglobin was dialysed for a week against distilled water, then against water containing $CO_2$ for one or two days, to remove bases combined with the protein, and finally re-dialysed against distilled water. Samples of the solution were then mixed with water, dilute HCl and dilute NaOH, and the $p_H$ values were determined with the Clark hydrogen electrode. The $CO_2$ contents of samples equilibrated with hydrogen were then measured. The calculations of the acid or base in combination with the protein were then made by formula 3, as described by Van Slyke *et al.* [1922] and Hastings, Van Slyke *et al.* [1924]. Within the range of concentrations of the protein and of electrolytes used in this work, the calculations of the acid bound made by these methods are in agreement with the more rigorous methods described by Sørensen *et al.* [1927]:

$$A_p c_p = [\text{Total anions}] - [\text{Total cations}] \qquad \ldots\ldots(3).$$

$A_p$ = equivalents of acid, *i.e.* hydrogen ions combined with one molecule of the protein. $c_p$ = molar concentration of the protein. The terms in square brackets denote the total concentrations of anions and cations in equivalents. Where anions are present in excess, as in mixtures of sodium chloride and protein chloride, $A_p$ is positive; where cations are present in excess, as in sodium proteinate, $A_p$ is negative, since the protein gives off hydrogen ions in alkaline solution.

Observations made by this method, recorded in Table XII and Fig. 6, indicate that the isoionic point, or more exactly, the $p_H$ value where $A_p = 0$, is approximately 7·4 at 0°.

In method B, devised to facilitate comparisons of isoionic and isoelectric points under the same conditions, the calculations of the acid bound are based on chemical analyses of the dialysate and of the protein solution, samples of the latter being removed from the membrane just before the measurement of the potentials. Experiments were carried out with a range of buffers, so that the point where $A_p = 0$ could be determined by interpolation, as shown in Fig. 5.

In calculations of $A_p$ for solutions of haemoglobin buffered with ammonium phosphates, it must be remembered that the analytical results are obtained in terms of total ammonia and total phosphoric acid. Appreciable amounts of un-ionised ammonium hydroxide are present if the $p_H$ is greater than 7·7 at 0°. Ammonium hydroxide in the dialysate is estimated from measurements of the total ammonia and of the $p_H$ by the application of the law of mass action, and it is assumed that the corrected concentrations, defined by formula 2, are the same in the protein solutions and in the dialysates. The total phosphoric acid in the $p_H$ range from 6 to 8 exists mainly in the form of the univalent ion $H_2PO_4$ and the bivalent $HPO_4$; the concentrations of free acid and of the trivalent ion can be neglected. The acid combined with protein can then be calculated without serious error by formula 4:

$$m_p A_p = 2[HPO_4]_c' + [H_2PO_4]_c' - [NH_4]_c' = [PO_4]_c'(1 + x') - [NH_4]_c' \qquad \ldots\ldots(4).$$

The symbol $[PO_4]_c'$ is used to represent the corrected concentration of total phosphate in the protein solution, in mols. per litre of solvent. $x'$ = the fraction of

the total phosphate in the protein solution in the bivalent form; an estimate of $x'$ can be obtained by the procedure below.

In the first place, $x''$, the proportion of the bivalent ion in the dialysate composed of the pure salts $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$, may be estimated by the application of the law of mass action to the measurements of hydrogen ion activities, or the concentrations of the ions $HPO_4$ and $H_2PO_4$ may be calculated

Table I. *Ion distributions and acid combined with haemoglobin.*

Sheep CO-haemoglobin, equilibrated with 0·02 *M* ammonium phosphate at 0°. Exp. 654. Osmotic pressure = 92·9 mm. Hg. Membrane potential = −0·57 mv. $p_{H''} = 7{\cdot}008$, $p_{H'}$ (calc.) = 6·998. C = 19·68. $m_p = 0{\cdot}003628$. $x' = 0{\cdot}50$. $A_p = 4{\cdot}96$. $f'/f''$, the ratio of the activity coefficients, computed by formula $f'.[NH_4]_c' = r_i f''.[NH_4]''$. In the dialysate $[HPO_4]'' = [NH_4]'' - [PO_4]''$, and $[H_2PO_4]'' = [PO_4]'' - [HPO_4]''$. $x'' = 0{\cdot}505$.

| | | | | Protein solution | | |
|---|---|---|---|---|---|---|
| Ion species | Dialysate concentration | Ideal distribution ratio $r_i$ | Corrected concentration, ideal | Concentration, observed | Corrected concentration, observed | $f'/f''$ |
| $NH_4$ | 0·03020 | 1/1·024 | 0·03093 | 0·02651 | 0·0327 | 0·945 |
| $PO_4$ (mol) | 0·02007 | — | 0·01935 | 0·02748 | 0·0339 | 0·571 |
| $HPO_4$ (mol) | 0·01013 | 1/1·05 | 0·00965 | — | 0·0169 | — |
| $H_2PO_4$ (mol) | 0·00994 | 1/1·024 | 0·00970 | — | 0·0170 | — |
| $PO_4$ (equivalent) | 0·03020 | — | 0·02900 | — | 0·0507 | — |

from analytical determinations of total ammonia and phosphoric acid, as shown in Table I, where the value of $x''$ is estimated by the formula

$$[NH_4]'' = (1 + x'')\,[PO_4]''.$$

The theoretical distribution ratio for each ion is then calculated by formula 5, a modification of the formula of Donnan and Allmand [1914] discussed by Adair and Adair [1934]:

$$r_i = \text{antilog}\,[-n_i E/54{\cdot}16] \qquad \ldots\ldots(5),$$

$r_i$ = ideal distribution ratio $= a_i'/a_i''$, where $a_i'$ and $a_i''$ denote the activities or effective concentrations of the diffusible ions in the protein solution and in the dialysate respectively; $n_i$ = the valence of the ion (negative for anions). The ideal concentration of each ion in the protein solution is then calculated by formula 6,

$$[NH_4]_c' \text{ (ideal)} = r_i\,[NH_4]'' \qquad \ldots\ldots(6).$$

The same notation is used in application of formula 6 to the other diffusible ions. $x'$ is estimated from these "ideal" concentrations, as shown in Table I. Calculations of $A_p$ based on these assumptions cannot be exact, but it is unlikely that the error is serious, since the titration curve obtained by this method agrees with results obtained by method A, as shown in Fig. 6.

In practical applications of formula 4, it is advisable to use solutions in which the protein concentration, $C$, exceeds 20 g. per 100 ml. solution, since an error of 0·00032 mol. per litre in the estimation of ammonia may cause an error of 0·01 $p_H$ unit in the determination of the isoionic point of a solution in which $C_v = 26{\cdot}8$ and $m_p = 0{\cdot}004$, whereas in a solution where $C_v = 2{\cdot}68$, the same error in the analyses causes a tenfold increase in the errors in the estimation of $A_p$ and of the isoionic point.

Gasometric analyses showed that the protein solutions dialysed against ammonium phosphate buffers may contain from 0·0001 to 0·0002 mol. of $CO_2$ per litre, concentrations which are too low to cause serious errors in the estimation of

the isoionic point of solutions where $C_v$ exceeds 20. In similar solutions dialysed against distilled water, the $CO_2$ content may be as much as 0·004 mol. per litre.

Method C is based on the hypothesis that if inorganic salts cause a difference between isoionic and isoelectric points, either by the combination of ions with the protein or by their effects on interionic forces, such differences must diminish as the salt concentration is reduced. A series of measurements of the isoelectric point was made and plotted against $\sqrt{\mu}$, the square root of the ionic strength of the dialysate, as shown in Fig. 7. If a straight line be drawn through the points and extrapolated to zero salt concentration, the $p_H$ value obtained is not far removed from the isoionic point determined by method B.

### 4. *The comparison of different preparations of haemoglobin.*

In order to determine whether the proteins prepared from the bloods of different individuals are identical [Barcroft, 1928], a number of preparations of CO-haemoglobin were made from sheep's blood by method 1 and equilibrated with ammonium phosphate buffer mixtures. Seven preparations gave a value for the isoelectric point of $p_H$ 7·06 ± 0·01 at 0°. The values for the remaining preparations ranged from 7·0 to 7·14, with one exception, which gave the value 6·75; this material had been exposed to high temperatures during centrifuging.

Table II. *Comparison of preparations of sheep CO-haemoglobin, made by method 1.*

Osmotic pressures in mm. Hg and membrane potentials in mv. at 0°, extrapolated for protein concentration $C = 21{\cdot}29$, $C_v = 26{\cdot}8$ and $m_p = 0{\cdot}004$, and for dialysate $(NH_4)_2HPO_4 = 0{\cdot}005\ M + NH_4H_2PO_4 = 0{\cdot}005\ M$, $p_H = 7{\cdot}09$ at 0°. Isoelectric points for 0·01 $M$ ammonium phosphates. Isoionic points estimated by method B, Section 3, except Exps. 594–601 and 613–622 by method C.

| Exp. no. | Osmotic pressure | Membrane potential | Isoelectric point | Isoionic point |
|---|---|---|---|---|
| 311 | — | −0·40 | 7·05 | 7·50 |
| 331–337 | 105 | −0·20 | 7·07 | 7·39 |
| 451–462 | 103 | −0·40 | 7·05 | 7·41 |
| 467–477 | 102 | −0·85 | 7·00 | 7·37 |
| 559–570 | 109 | +0·50 | 7·14 | — |
| 577–588 | 109 | +0·40 | 7·13 | 7·42 |
| 594–601 | 107 | −0·35 | 7·05 | 7·56 |
| 613–622 | 101 | −2·93 | 6·75 | 7·34 |
| 623 | — | −0·20 | 7·07 | — |
| 654, 655 | 107 | −0·20 | 7·06 | 7·63 |
| 641 | 107 | −0·20 | 7·06 | — |
| N. H. | 106 | +0·30 | 7·11 | 7·56 |
| S. | 107 | +0·30 | 7·13 | 7·56 |

The experimental determinations of the isoelectric and isoionic points represent the total protein present in the preparations, which contain traces of other derivatives in addition to CO-haemoglobin. Oxyhaemoglobin is present in all solutions containing dissolved air, but the titration curves of Hastings, Sendroy *et al.* [1924] and measurements of cataphoresis [Svedberg, 1933] indicate that replacement of oxygen by carbon monoxide causes no change in the isoelectric or isoionic points. The isoionic point is affected by reduction, but the concentration of reduced haemoglobin is very small in the presence of carbon monoxide at 0°. Methaemoglobin or other derivatives which do not combine reversibly with oxygen and carbon monoxide are formed during the preparation of haemoglobin. The uncrystallised haemoglobin made by method 1 combined with 1·27 ± 0·03 ml. CO per g. protein; crystalline haemoglobin prepared by method 3, combined with

1·21 ± 0·04 ml. CO per g. protein. If the difference between these figures and the theoretical value of 1·34 ml. per g. be attributed to methaemoglobin, the proportion of this protein is about 5 % in the uncrystallised and 10 % in the crystallised preparations. If the protein be completely changed into methaemoglobin, the isoelectric point is increased from $p_H$ 6·92 to 7·04 [Svedberg, 1933], whereas the isoionic point is increased by 0·15 ± 0·05 $p_H$ unit [Hastings *et al.*, 1924]. The variations in isoelectric and isoionic points attributable to the formation of 10 % of methaemoglobin are 0·006 and 0·015 $p_H$ unit respectively.

Unpublished experiments have shown that the denaturation of haemoglobin may yield products that are more acidic or more basic than native haemoglobin, and it is conceivable that the formation of soluble derivatives of a type intermediate between native and denatured protein might account for the variations in the isoionic points of haemoglobin shown in Table II and by other workers. The possibility that there are slight irreversible changes in the protein is indicated furthermore by the observation that a preparation kept at 0° for 16 months showed an increase in isoelectric point from 7·00 to 7·07, without appreciable change in the CO capacity. It is possible that the anomaly in the isoelectric behaviour of gelatin described by Pauli and Valkó [1933] can be accounted for by differences in individual preparations.

It is unlikely that the solutions used in this work contained appreciable amounts of completely denatured haemoglobin, since the protein was highly soluble at the isoelectric point and formed a wholly crystalline solid phase on addition of ammonium sulphate. The osmotic pressures, moreover, were all within 4 % of the mean value of 105·5 mm. at 0° for a solution with $m_p = 0·004$, without making corrections for the variations of pressure with the charge on the protein ions, which would reduce the differences observed.

Comparison of crystallised and uncrystallised haemoglobin prepared from the same blood, recorded in Table III, shows that the isoelectric point of the crystallised preparations is at a more alkaline $p_H$. No appreciable difference

Table III. *Isoelectric points of crystallised and uncrystallised haemoglobin of sheep.*

Dialysates, ammonium phosphates at 0°. Each number refers to blood of a different individual.

| No. | Molarity of buffer | Total haemoglobin | Crystallised haemoglobin | Recrystallised haemoglobin | Mother-liquor |
|---|---|---|---|---|---|
| 1 | 0·01 | 7·03 | 7·12 | — | — |
| 2 | 0·01 | 7·14 | 7·18 | 7·18 | 7·19 |
| 3 | 0·02 | 6·86 | 6·91 | — | 6·91 |

could be detected in measurements of nitrogen content, hydration, osmotic pressure, refraction or density. In the crystallised preparations made by method 3, described above, the amount of methaemoglobin formed, as estimated by the fall in CO capacity after crystallisation, is about 5 %; the variations in isoelectric point, ranging from 0·04 to 0·1, exceed the changes attributable to methaemoglobin. It is unlikely that the change is due to the existence of two different forms of haemoglobin, or to the removal of an acidic impurity by crystallisation, since preparations of concentrated mother-liquor appear to have the same isoelectric point as crystallised or recrystallised protein, as shown in Table III. If fractionation takes place, the acidic substances should be more concentrated in the mother-liquor than in the total protein or the crystalline fraction. From these experiments and from the observation that solutions of crystallised haemoglobin may yield traces of precipitate at 0°, it may be inferred

that the change in isoelectric points is due to the action of ammonium sulphate on the protein.

Dr K. O. Pedersen kindly examined two solutions, Exp. 2, total and crystallised haemoglobin in Table III (sent to Upsala in ice in a thermos flask) and found that the bulk of the protein, with a light absorption of wave-length 5–600 $m\mu$, was homogeneous [Pedersen, 1933]. A trace of a substance with a different mobility was detected, a larger amount of this being present in the crystallised than in the uncrystallised material. The isoelectric point was at $p_H$ 7·09 for both preparations, measured by cataphoresis at 20° with $KH_2PO_4$-$Na_2HPO_4$ buffers of 0·02 ionic strength. In view of Dr Pedersen's results, it seems that the haemoglobins made by methods 1 and 3 are much more nearly homogeneous than the preparations obtained by Geiger [1931], who reported the presence of relatively large amounts of two different fractions, isolated by the method of cataphoresis.

Measurements were made to determine whether ether has an injurious effect on the protein. The isoelectric points of preparations made with and without ether are not identical, but the mean difference found of 0·05 $p_H$ unit is not excessive. It is probable that the preparations made with ether give a better approximation to a pure, unaltered protein, since the material obtained without the use of ether was kept at room temperature for a longer time, in efforts to remove stroma-protein by centrifuging. Measurements of the carbon dioxide dissociation curves of human haemoglobin showed no evidence of irreversible changes in the protein caused by the addition of ether during the process of laking [Adair, 1925, 1].

The comparisons of different preparations of sheep CO-haemoglobin have not been restricted to the measurements given in Tables II and III. The evidence as a whole has shown that although the absolute values of the isoelectric and isoionic points are affected by the methods of preparation, there are properties of the protein which are practically constant, including the changes in the membrane potentials and the valences with the $p_H$, the effects of salts on the isoelectric point and the effects of the protein on the activities of the diffusible ions. In the following sections, which are concerned with the properties which are more nearly constant, detailed comparisons of different preparations have not been recorded.

It is possible that the variations in the isoelectric and isoionic points of individual preparations of sheep haemoglobin would be less marked in the case of ox haemoglobin, since the globin moiety of this protein is more stable.

### 5. *Haemoglobin and ammonium phosphate.*

In the investigation of the equilibrium of sheep CO-haemoglobin and ammonium phosphate buffers, recorded in Tables IV, V, VI, VII and VIII, a single preparation of protein was used in each series of experiments, in order to obtain comparable results in studies of the effects of hydrogen ions and salts on the membrane potentials. If a curve be constructed from data obtained with more than one preparation, allowances must be made for differences between the preparations, referred to in Section 4. With the exception of the experiments with crystalline haemoglobin, given in Table VIII, the haemoglobin was prepared by method 1. Estimations of the isoelectric points of the preparations are included in Table II.

The solutions of ammonium phosphates $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$, used in the dialysis fluids, were prepared from recrystallised salts or from an analysed sample of A.R. $(NH_4)_2HPO_4$, which gave theoretical values for ammonium and

phosphoric acid. Glass-distilled and boiled-out water was used and the solutions were stored at 0°. In estimations of the ionic strength and other calculations, it has been assumed provisionally that the salts are fully ionised in accordance with recent work on strong electrolytes [Debye and Hückel, 1923]. On this assumption, the ionic strength $\mu$, defined as half the sum of the concentrations of the ions in the dialysate, multiplied by the squares of their valences, is equal to $[NH_4H_2PO_4]'' + 3[(NH_4)_2HPO_4]''$.

Table IV gives the experimental data required for the determination of the isoelectric point by interpolation (method 2, Section 2) as shown in Fig. 1, in

Table IV. *CO-haemoglobin and ammonium phosphate buffers of constant molarity.*

Exps. 451–462, preparation 1931, 4, isoelectric point 7·05. Exps. 467–477, preparation 1931, 5, isoelectric point 7·00. Dialysates 0·01 *M* ammonium phosphates. $C$ = g. dry protein in 100 ml. solution. $p$ = osmotic pressure in mm. Hg at 0°. $p_H{}'' = p_H$ value of dialysate. $E$ = membrane potential in mv. at 0°. $[NH_4]''$ = total ammonia in mols. per litre in dialysate. $[NH_4]'$ = total ammonia in protein solution. $[PO_4]''$ = total phosphoric acid in dialysate. $[PO_4]'$ = total phosphoric acid in protein solution.

| Exp. no. | $C$ | $p$ | $p_H{}''$ | $E$ | $[NH_4]''$ | $[PO_4]''$ | $[NH_4]'$ | $[PO_4]'$ |
|---|---|---|---|---|---|---|---|---|
| 451 | 20·6 | 142·6 | 6·30 | +7·93 | 0·0133 | 0·00997 | 0·0089 | 0·02663 |
| 452 | 21·2 | 107·2 | 6·94 | +1·03 | 0·0136 | 0·00969 | 0·0131 | 0·01739 |
| 454 | 21·0 | 100·7 | 7·10 | −0·38 | 0·0150 | 0·00997 | 0·0155 | 0·01497 |
| 455 | 21·4 | 103·4 | 7·11 | −0·39 | 0·0154 | 0·01000 | 0·0152 | 0·01505 |
| 456 | 21·6 | 98·9 | 7·26 | −1·83 | 0·0161 | 0·00999 | 0·0152 | 0·01366 |
| 458 | 20·2 | 93·8 | 7·42 | −2·70 | — | 0·01010 | 0·0182 | 0·01126 |
| 459 | 20·7 | 100·9 | 7·59 | −3·67 | 0·0167 | 0·01000 | 0·0193 | 0·00930 |
| 460 | 20·7 | 98·5 | 7·53 | −3·35 | 0·0176 | 0·01005 | — | — |
| 461 | 19·9 | 99·6 | 7·68 | −4·57 | 0·0183 | 0·01010 | 0·0239 | 0·00861 |
| 462 | 19·3 | 152·7 | 8·44 | −9·75 | 0·0215 | 0·01007 | 0·0331 | 0·00452 |
| 467 | 21·4 | 124·0 | 6·67 | +3·17 | 0·0121 | 0·00984 | 0·0102 | 0·02180 |
| 469 | 21·5 | 109·7 | 6·94 | +0·62 | 0·0141 | 0·00980 | 0·0141 | 0·01706 |
| 471 | 21·9 | 107·1 | 7·09 | −0·88 | 0·0145 | 0·00982 | 0·0142 | 0·01510 |
| 473 | 21·8 | 105·6 | 7·26 | −1·98 | 0·0152 | 0·00999 | 0·0147 | 0·01348 |
| 474 | 20·8 | 96·9 | 7·37 | −2·45 | 0·0166 | 0·00996 | 0·0170 | 0·01190 |
| 475 | 21·8 | 105·4 | 7·40 | −3·07 | 0·0160 | 0·00993 | 0·0181 | 0·01123 |
| 476 | 21·8 | 110·8 | 7·54 | −3·93 | 0·0182 | 0·01006 | 0·0199 | 0·01008 |
| 477 | 21·7 | 166·9 | 8·23 | −9·20 | 0·0224 | 0·00990 | 0·0301 | 0·00506 |

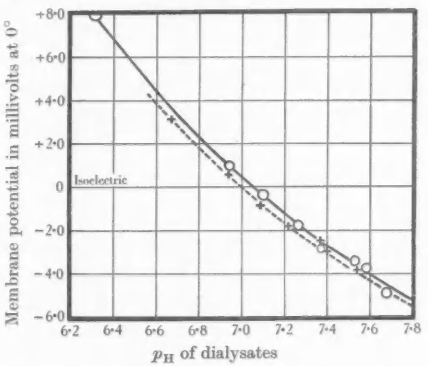



Fig. 1. Isoelectric point of sheep CO-haemoglobin, method 2, Section 2. Dialysates 0·01 *M* ammonium phosphates. Membrane potentials for 21·29 % protein solutions ($m_p$ = 0·004); extrapolated from data in Table IV. Circles, preparation 1931, 4; crosses, preparation 1931, 5.

systems in which the molarity of the dialysate is kept constant. The measurements of the concentration of ammonia and phosphoric acid included in this table were used in the calculations of the acid bound by the protein, the valence of the protein (formula 9) and the isoionic point (method B, Section 3), shown in Fig. 5. Values of the ratio of the activity coefficients $f'/f''_{PO_4}$, based on data in Table IV, are recorded in Table XV. This ratio represents a mean value for the ions $HPO_4$ and $H_2PO_4$, computed from the formula

$$f'/f''_{PO_4} = [PO_4]_c' \text{ (ideal)}/[PO_4]_c' \text{ (observed)}.$$

Similar values were obtained with other preparations. In the case of ammonium ions, the results for $f'/f''_{NH_4}$, computed from Table IV, are more irregular and smaller than values computed from Table VIII. It is probable that the difference is due to errors in the determinations of ammonia included in Table IV. In all cases the protein causes a much greater diminution in the activities of the anions than of the cations. Slight differences in the same direction have been observed by Hastings *et al.* [1927] and by Adair [1928], but their experiments were made at a higher salt concentration, under conditions less favourable for the demonstration of specific effects due to the protein.

The effects of variation in the hydrogen ion concentration with buffers of constant ionic strength are shown in Table V. The buffer value of the solutions of

Table V. *Effects of variations of* $p_H$ *at constant ionic strength.*

Sheep CO-haemoglobin and ammonium phosphate buffers at 0°. Exps. 559–570, preparation 1932, 1; Exps. 577–588, preparation 1932, 4. Isoelectric points for both preparations are 7·34, 6·46 and 6·15 with dialysates of ionic strengths 0·01, 0·10 and 0·20 and phosphate concentrations of 0·04, 0·072 and 0·138 respectively.

| Exp. no. | Ionic strength | Molarity $PO_4$ | $p_{H''}$ | $C$ | $p$ | $E$ | $E^*$ |
|---|---|---|---|---|---|---|---|
| 559 | 0·01 | 0·008 | 6·40 | 19·8 | 147·1 | +9·25 | +9·10 |
| 560 | ,, | 0·006 | 6·90 | 20·7 | 121·8 | +5·0 | +5·05 |
| 561 | ,, | 0·005 | 7·13 | 20·8 | 109·8 | +2·9 | +2·80 |
| 562 | ,, | 0·004 | 7·42 | 20·5 | 94·7 | −1·44 | — |
| 563 | 0·10 | 0·08 | 6·00 | 21·2 | 117·9 | +0·63 | — |
| 564 | ,, | 0·06 | 6·58 | 20·7 | 101·5 | −0·2 | −0·12 |
| 565 | ,, | 0·05 | 6·89 | 20·9 | 102·7 | −0·7 | — |
| 566 | ,, | 0·04 | 7·36 | 20·8 | 103·5 | −1·25 | −1·40 |
| 567 | 0·20 | 0·16 | 5·85 | 21·2 | 115·4 | +0·08 | +0·05 |
| 568 | ,, | 0·12 | 6·45 | 20·8 | 108·0 | −0·18 | −0·10 |
| 569 | ,, | 0·10 | 6·77 | 20·6 | 103·0 | −0·40 | — |
| 570 | ,, | 0·08 | 7·27 | 21·1 | 108·6 | −1·00 | −0·80 |
| 577 | 0·01 | 0·008 | 6·33 | 20·8 | 169·5 | +9·56 | — |
| 578 | ,, | 0·006 | 6·82 | 21·5 | 132·5 | — | — |
| 579 | ,, | 0·005 | 7·11 | 21·6 | 116·4 | +2·47 | — |
| 580 | ,, | 0·004 | 7·47 | 21·7 | 106·0 | −2·26 | — |
| 581 | 0·10 | 0·08 | 6·00 | 21·5 | 120·8 | +0·75 | — |
| 582 | ,, | 0·06 | 6·57 | 21·5 | 111·5 | −0·23 | — |
| 583 | ,, | 0·05 | 6·88 | 21·5 | 109·3 | −0·65 | — |
| 584 | ,, | 0·04 | 7·37 | 20·5 | 102·3 | −1·44 | — |
| 585 | 0·20 | 0·16 | 5·84 | 21·4 | 116·5 | +0·4 | — |
| 586 | ,, | 0·12 | 6·43 | 21·1 | 109·4 | −0·37 | — |
| 587 | ,, | 0·10 | 6·77 | 21·0 | 108·0 | −0·60 | — |
| 588 | ,, | 0·08 | 7·28 | 21·0 | 108·4 | −0·93 | — |

$E^*$. Supplementary experiments with stoppered membranes, at the same $p_H$ and protein concentration.

ionic strength 0·01 is low, and the final $p_H$ values of the dialysates and the standard solutions are not identical; analyses are given in Table VI. The pro-

Table VI. *Concentrations of ions in protein solution and dialysate.*

| Exp. no. | $[NH_3]''$ | $[PO_4]''$ | $[NH_3]$ | $[PO_4]'$ |
|---|---|---|---|---|
| 577 | 0·0102 | 0·00871 | 0·00676 | 0·02400 |
| 579 | 0·0076 | 0·00496 | 0·00703 | 0·01044 |
| 580 | 0·0067 | 0·00393 | 0·00932 | 0·00560 |

portions of the total phosphate in the buffer solution in the bivalent form $x''$ can be computed by the formula $\mu = [PO_4]''(1+2x'')$, from the values given in Table V.

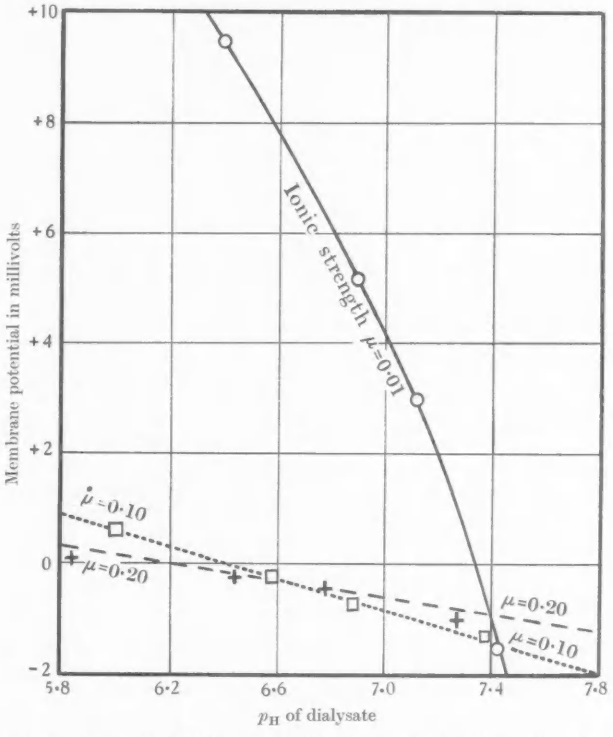



Fig. 2. Isoelectric point of sheep CO-haemoglobin. Dialysates ammonium phosphate buffers of ionic strengths 0·01, 0·10 and 0·20. Membrane potentials for protein solutions of $m_p = 0·004$, extrapolated from data in Table V.

The membrane potentials recorded in Fig. 2 show that an increase in salt concentration displaces the isoelectric point towards the acid side, and reduces the slopes of the curves, in accordance with formula 8, a result which indicates that the membrane potential varies inversely as the ionic strength of the dialysate. The intersections of the curves shown in Fig. 2 are eliminated when the apparent valences are calculated as shown in Fig. 6.

In order to obtain values of the isoelectric point at a greater number of salt concentrations with each preparation of haemoglobin, series of experiments

were made by method 3, Section 2. The results for two preparations are given in Table VII. It will be seen that if the molarities exceed 0·01, there is little difference between the $p_H$ values of the stock buffers and those of the final

Table VII. *Effects of increasing salt concentration on the isoelectric point.*

Sheep CO-haemoglobin, dialysates ammonium phosphates at 0°. Exps. 594–601, preparation 1932, 8; Exps. 613–622, preparation 1932, 10; isoelectric points estimated by method 3, Section 2, on the approximate assumption that the ionic strengths of the dialysates are equal to those of the standard buffer solutions, which contained equal proportions of $(NH_4)_2HPO_4$ and $NH_4H_2PO_4$.

| | Standard buffer solution | | | Dialysate | | | | |
|---|---|---|---|---|---|---|---|---|
| Exp. no. | Ionic strength | Molarity $PO_4$ | $p_H$ | $p_{H''}$ | $C$ | $p$ | $E$ | Isoelectric point |
| 594 | 0·01 | 0·005 | 7·11 | 7·23 | 21·2 | 103·4 | 0·00 | 7·23 |
| 595 | 0·01 | 0·005 | 7·11 | 7·16 | 21·04 | 109·6 | +1·46 | 7·27 |
| 596 | 0·02 | 0·010 | 7·09 | 7·10 | 20·5 | 101·6 | −0·47 | 7·03 |
| 597 | 0·02 | 0·010 | 7·09 | 7·13 | 21·6 | 108·4 | −0·23 | 7·10 |
| 600 | 0·04 | 0·020 | 7·02 | 7·03 | 21·4 | 106·7 | −0·57 | 6·90 |
| 601 | 0·04 | 0·020 | 7·02 | 7·01 | 21·08 | 105·6 | −0·69 | 6·85 |
| 614 | 0·01 | 0·005 | 7·11 | 7·20 | 20·3 | 92·5 | −3·23 | 6·97 |
| 615 | 0·02 | 0·010 | 7·09 | 7·13 | 20·2 | 92·2 | −2·92 | 6·76 |
| 616 | 0·02 | 0·010 | 7·09 | 7·12 | 20·4 | 93·7 | −2·77 | 6·77 |
| 617 | 0·04 | 0·020 | 7·02 | 7·04 | 20·1 | 94·0 | −2·22 | 6·51 |
| 619 | 0·07 | 0·035 | 6·95 | 6·96 | 20·3 | 94·9 | −1·69 | 6·28 |
| 620 | 0·07 | 0·035 | 6·95 | 6·95 | 20·3 | 95·2 | −1·72 | 6·26 |
| 621 | 0·10 | 0·050 | 6·89 | 6·89 | 20·3 | — | −1·38 | 6·11 |
| 622 | 0·10 | 0·050 | 6·89 | 6·89 | 20·3 | — | −1·32 | 6·14 |

dialysates. Although the absolute values of the isoelectric points for preparation 1932, 10 differ from normal values by 0·3 $p_H$ unit, the changes caused by an increase in the concentration of phosphates are very similar to the results obtained with other preparations.

Measurements of the distribution of ions in solutions with increasing phosphate concentrations are given in Table VIII. Calculations of the ratio $f'/f''_{PO_4}$ based on

Table VIII. *Membrane potentials and ion distributions at different salt concentrations.*

Sheep CO-haemoglobin and ammonium phosphates at 0°. Exps. 598, 599, crystallised protein preparation 1932, 8; Exps. 642, 645, 646, crystallised preparation 1932, 6 B. Remaining Exps., uncrystallised haemoglobin from 6 preparations. Exps. C 23 and C 24, from same blood as Exps. C 3 and C 6, Table X. $f'/f''_{NH_4}$ usually exceeds 0·95.

| Exp. no. | $C$ | $p$ | $p_{H''}$ | $E$ | $[NH_4]''$ | $[PO_4]''$ | $[NH_4]'$ | $[PO_4]'$ |
|---|---|---|---|---|---|---|---|---|
| 598 | 21·02 | 104·8 | 7·08 | +0·61 | 0·01500 | 0·01000 | — | — |
| 599 | 21·16 | 108·9 | 7·09 | +0·97 | 0·01500 | 0·01000 | — | — |
| 645 | 21·40 | 110·2 | 7·08 | +0·68 | 0·01510 | 0·00996 | — | 0·01730 |
| 642 | 21·67 | 108·1 | 7·02 | −0·47 | 0·02993 | 0·02010 | 0·0269 | 0·02770 |
| 646 | 21·92 | 109·1 | 6·95 | −0·69 | 0·05150 | 0·03440 | 0·0426 | 0·04370 |
| NH 6 | 19·38 | — | 7·08 | +0·63 | 0·01503 | 0·00999 | 0·0126 | 0·01559 |
| NH 47 | 21·10 | — | 7·08 | +0·69 | 0·01503 | 0·00999 | 0·0117 | 0·01601 |
| C 23 | 20·20 | 98·4 | 7·08 | +0·33 | 0·01485 | 0·00990 | 0·0116 | 0·01540 |
| C 24 | 19·90 | 95·5 | 7·08 | +0·30 | 0·01520 | 0·01010 | 0·0124 | 0·01610 |
| 640 | 21·94 | 113·1 | 7·05 | −0·86 | 0·03057 | 0·01993 | 0·0255 | 0·02940 |
| 655 | 19·70 | 91·4 | 7·02 | −0·61 | 0·03030 | 0·02000 | 0·0261 | 0·02750 |
| 641 | 21·90 | 110·9 | 7·04 | −0·81 | 0·03053 | 0·01970 | 0·0278 | 0·02930 |
| 326 | 16·50 | 73·9 | 6·76 | −0·10 | 0·22390 | 0·13830 | 0·1952 | 0·14500 |
| 611 | 20·30 | 124·0 | 6·30 | — | 1·48000 | 0·99100 | 1·1700 | 0·80730 |

these measurements for crystalline and for uncrystallised haemoglobin are almost identical. It may be noted that the phosphate determinations in Exps. 640 and 641 are less reliable than in Exp. 655.

### 6. *Haemoglobin and sodium phosphates.*

Preliminary measurements of the effects of sodium salts on the isoelectric points of two preparations of sheep CO-haemoglobin are given in Table IX. In

Table IX. *Effects of different cations and anions on the isoelectric point of sheep CO-haemoglobin at 0°.*

Molarities of anions in dialysates; $HPO_4$, 0·005; $H_2PO_4$, 0·005; Cl 0·05. Isoionic points, preparation 1933, 11 = 7·55; preparation 1934, 1 = 7·58; preparation 1934, 2 = 7·57.

| Cation species | Cation molarity | Anion species | Isoelectric point: Preparation 1933, 11 | Preparation 1934, 1 | Preparation 1934, 2 |
|---|---|---|---|---|---|
| $NH_4$ | 0·015 | $HPO_4$, $H_2PO_4$ | — | 7·15 | 7·13 |
| Na | 0·015 | $HPO_4$, $H_2PO_4$ | 7·05 | 7·10 | — |
| Na | 0·065 | $HPO_4$, $H_2PO_4$, Cl | 6·93 | 7·01 | — |
| Na + K | 0·015 | $HPO_4$, $H_2PO_4$ | — | — | 7·10 |

the second experiment (Jan. 1934), equilibration was carried out for a prolonged period during which slight changes took place in the protein. The final CO capacities were from 93 to 95·4 % of the theoretical values. From these results, the isoelectric point with 0·01 *M* sodium phosphates appears to be slightly on the acid side of that obtained with ammonium phosphates.

The addition of 0·05 mol. of sodium chloride to the sodium phosphate buffer, increasing the ionic strength from 0·02 to 0·07, alters the isoelectric point to a more acid reaction. The difference of 0·09 to 0·12 $p_H$ unit is in the same direction as that observed when the ionic strengths of ammonium phosphate buffers are increased over the same range, but the change in isoelectric point with ammonium phosphates is about 0·46 $p_H$ unit. There is little doubt that this difference between the effects of chlorides and phosphates on the isoelectric point is correlated with the observation that the diminution of the activities of the ions caused by haemoglobin is greater for phosphates than for chlorides, as shown by Adair [1928] and by the measurements recorded in Table X, which includes some

Table X. *Membrane equilibrium of sodium, chloride and phosphate ions.*

Sheep CO-haemoglobin at 0°. Exps. B, C, E, F, preparation 1933, 11, dialysate sodium phosphates. Exps. C 3, C 6, preparation 1934, 2 S, dialysate 0·005 *M* $Na_2HPO_4$ + 0·005 *M* $KH_2PO_4$. Values for total base under heading Na. Concentrations of ions in mols. per l.

| Exp. no. | B | C | E | F | C 6 | C 3 |
|---|---|---|---|---|---|---|
| $[Cl]''$ | 0 | 0 | 0·0499 | 0·0500 | 0 | 0 |
| $[PO_4]''$ | 0·00996 | 0·00986 | 0·00998 | 0·0099 | 0·00975 | 0·0099 |
| $[Na]''$ | 0·0149 | 0·0171 | 0·0618 | 0·0647 | 0·0147 | 0·0146 |
| $[Cl]'$ | 0 | 0 | 0·0480 | 0·0460 | 0 | 0 |
| $[PO_4]'$ | 0·01577 | 0·01168 | 0·0126 | 0·0113 | 0·0152 | 0·0146 |
| $[Na]'$ | 0·01156 | 0·0154 | 0·0479 | 0·0521 | 0·0125 | 0·0126 |
| C | 22·4 | 23·6 | 23·9 | 22·23 | 20·04 | 17·75 |
| $p$ | 115·6 | 125·8 | 134·4 | 115·8 | 94·9 | 76·0 |
| $p_{H''}$ | 7·116 | 7·444 | 6·949 | 7·288 | 7·127 | 7·132 |
| $E$ | −0·65 | −3·05 | −0·07 | −0·98 | −0·08 | −0·31 |
| $f'/f''_{Na}$ | 1·04 | 0·97 | 0·99 | 1·03 | 0·96 | 0·97 |
| $f'/f''_{PO_4}$ | 0·48 | 0·53 | 0·61 | 0·65 | 0·52 | 0·55 |
| $f'/f''_{Cl}$ | — | — | 0·80 | 0·82 | — | — |

approximate estimates of the effects of the protein on the activity of the sodium ions, subject to an error of not less than 5 %.

The continuous line marked $PO_4$ 0·01 in Fig. 3 is constructed from the results of experiments with 0·01 *M* ammonium phosphates (Nos. 467–477), by adding 0·13 $p_H$ unit to the $p_H$ values in Table IV, to facilitate comparisons with

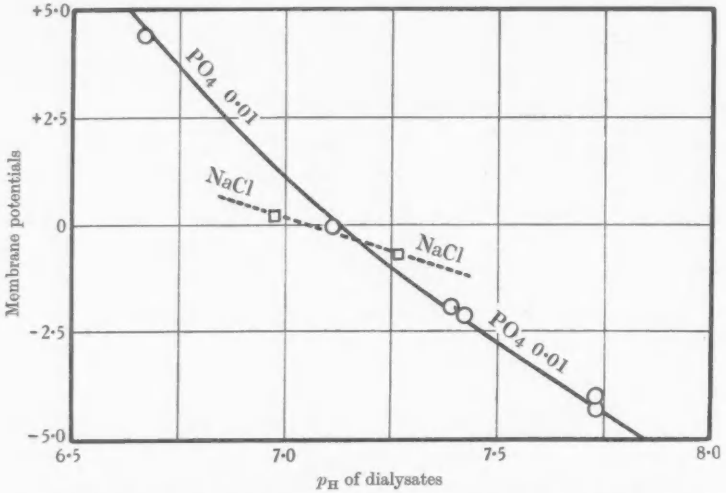



Fig. 3. Effect of sodium salts on membrane potentials. Sheep CO-haemoglobin at 0° and $m_g$=0·004, preparation 1934, 1. Circles, dialysate 0·01 *M* sodium phosphates; squares 0·01 *M* sodium phosphates + 0·05 *M* sodium chloride. Continuous curve, results with ammonium phosphates with a constant correction applied to the $p_H$ values.

the membrane potentials with sodium phosphates. The observed points are close to the calculated curve, and it seems that the general forms of the curves given by sodium and by ammonium phosphates are almost identical over the range investigated. The broken line, marked NaCl, shows that the addition of 0·05 mol. of sodium chloride to the dialysate diminishes the slope of the curves.

### 7. *Equilibrium of protein crystals, protein solution and dialysate.*

Sørensen [1917] showed that crystals of egg-albumin may contain an amount of combined ammonia or sulphuric acid approximately equal to the amounts combined with the protein solution. His method gives the difference between the ammonia and sulphuric acid, but it cannot give the absolute amounts of acids or bases present in the crystals. The method described below was devised in order to study the absolute amounts. A protein sparingly soluble in the region of the isoelectric point, such as horse CO-haemoglobin, was crystallised by dialysis against 0·01 *M* ammonium phosphate buffer for about 14 days at 0°. A 5 ml. density bottle was fitted to a 10 ml. tube of hard glass, and the vessel so obtained was filled with the crystal suspension and left to sediment at 0°. A sample of mother-liquor was obtained from the tube and filtered if necessary. After sufficient time had been allowed for sedimentation,

the density bottle, containing both crystals and mother-liquor, was stoppered and weighed, and the contents were washed out into a 100 ml. volumetric flask. If necessary, sodium hydroxide was added to facilitate dissolution of the protein crystals, care being taken that the alkalinity was not sufficient to cause the evolution of ammonia. Analyses were made for protein, ammonia and phosphoric acid content. The corrected concentrations have been calculated by formula 2, and the composition of the crystals estimated by graphical methods.

In an indirect analysis of this type, necessitated by the fact that the crystals cannot be separated from their mother-liquor without alteration in their composition, the experimental errors may be considerable, and the number of observations recorded in Table XI is insufficient for the establishment of definite formulae, but it is evident that of the three hypotheses that the crystals are composed of pure protein, pure protein salt, or protein *plus* acid *plus* ammonia,

Table XI. *Equilibrium of crystals, mother-liquor and dialysate. Horse CO-haemoglobin.*

The phase designated crystal is a mixture of crystals and mother-liquor.

| Exp. no. | Phase | $m_p$ | $[NH_4]_c$ | $[PO_4]_c$ |
|---|---|---|---|---|
| 5B 5 | Dialysate | — | 0·0167 | 0·0101 |
| „ | Filtrate | 0·00065 | 0·0189 | 0·0108 |
| „ | Crystal | 0·00500 | — | 0·0179 |
| SB 3 | Dialysate | — | 0·0173 | 0·0097 |
| „ | Filtrate | 0·00131 | 0·0207 | 0·0117 |
| „ | Crystal | 0·00229 | 0·0240 | 0·0142 |
| „ | Pressed crystals | 0·00850 | — | 0·0283 |
| SB 2 | Dialysate | — | — | 0·0099 |
| „ | Filtrate | 0·00160 | — | 0·0177 |
| „ | Crystal | 0·00225 | — | 0·0148 |
| SB 1 | Dialysate | — | 0·0145 | 0·0096 |
| „ | Filtrate | 0·00221 | 0·0167 | 0·0165 |
| „ | Crystals | 0·00382 | 0·0225 | 0·0213 |
| SB 4 | Dialysate | — | — | 0·0100 |
| „ | Filtrate | 0·0024 | 0·0140 | 0·0109 |
| „ | Crystals | 0·0061 | 0·0196 | 0·0250 |

as stated in formula 7, the third is correct. Cohn and Prentiss [1927] measured the solubility of haemoglobin in more concentrated buffers, of ionic strength 0·04 to 1·0, and state that their results suggest the identity of the "saturating body" in the systems studied.

$$Pr\,(H_2O)_w\,(NH_4)_b\,(PO_4)_a \qquad \text{.....(7).}$$

The terms $a$ and $b$ appear to be of the same order of magnitude for both dissolved and crystalline protein, and both vary with the hydrogen ion concentration. It has been assumed that $w = 800$ mol. of water per 67,000 g. dry protein. The higher value for hydration given by Sørensen and Sørensen [1933] would yield larger values for $a$ and $b$. In the isoionic region ($p_H$ 7·4) the term $a$ is nearly 2 mols. of phosphoric acid per mol. of haemoglobin.

Evidence for the presence of water in the crystalline phase was obtained from measurements of density. The specific volume of anhydrous haemoglobin at 20°, determined by the method used by Svedberg [1930], is 0·75 ml., and the density 1·333. Direct measurements made in this work showed that the crystals remained in suspension in media of densities from 1·267 to 1·269 at 20°. Mixtures of half-saturated ammonium sulphate and sucrose were used for these experiments, rather than the solutions including calcium chloride usually employed to

determine the density of crystals by this method, in order to reduce the risks of alterations in the protein.

The value 1·259 is calculated on the assumption based on chloride distribution measurements [Adair, 1928] that 1 g. dry protein combines with 0·2145 g. water and that 1·2145 g. protein hydrate occupies the sum of the volumes 0·75 ml. + 0·215 ml. attributed to the dry protein and the water respectively. The difference between the values 1·268 ± 0·001 and 1·259 may be accounted for by the effect of the sucrose and ammonium sulphate on the vapour pressure of water and the hydration of the protein.

Svedberg's calculations of molecular weights of proteins from ultracentrifugal measurements are based on the specific volume of the dry protein; recalculations based on the density 1·259 give considerably higher values, but there is no fundamental discrepancy because the lower density refers to the protein hydrate. It may be noted that in the cases of haemoglobin and serum-albumin, estimations of the "molar friction" (or of the radius of the particles) from the sedimentation velocities and from the molecular weights [Svedberg, 1930] are in much closer agreement if the results are expressed in terms of the mass and density of the protein hydrate.

In order to minimise the risk of error due to imperfect equilibration, some experiments on the salt distribution in crystals were made by a modified method, in which pure crystals were first formed by dialysis against distilled water; dialysis was then continued against buffer solutions. It was found that the same excess of ammonia and phosphate was present in the crystalline phase as in the systems in which crystallisation was brought about by dialysis against buffer solution.

8. *Relationship between osmotic pressure and isoionic and isoelectric points.*

In solutions of haemoglobin equilibrated with distilled water, containing only slight traces of electrolytes such as ammonia and carbon dioxide, the isoelectric and isoionic points and the minimum of osmotic pressure should be almost identical. At the minimum of pressure published by Adair [1928], the uncorrected $p_H$ of the dialysate, measured colorimetrically at room temperature, is 6·8 ± 0·2.

In systems of this type, measurements of hydrogen ion concentration are more reliable if they are made on the protein solution rather than on the dialysate, which is an unbuffered solution of low conductivity. Table XII records measurements made on haemoglobin solutions at 0°, using the hydrogen electrode. The observations are incomplete, since measurements of the $CO_2$ content of the protein solution before electrometric measurements are required,

Table XII. *Osmotic pressures, $p_H$ values, $CO_2$ content and isoionic point of haemoglobin.*

Sheep CO-haemoglobin, preparation 1931, 1. Dialysate distilled water at 0°. $p$ = extrapolated osmotic pressures for $m_p = 0{\cdot}0035$. $CO_2$ in millimols per l.

| Exp. no. | $p_{H'}$ | $p$ | $C$ | $p$ observed | $CO_2$ | Isoionic point |
|---|---|---|---|---|---|---|
| 554 | 6·83 | 163·0 | 16·08 | 132·3 | — | — |
| 551 | 7·24 | 112·2 | 18·65 | 108·7 | 2·8 | 7·37 |
| 479 | 7·27 | 112·0 | 20·70 | 124·0 | — | — |
| 553 | 7·32 | 103·7 | 18·58 | 100·3 | 1·9 | 7·40 |
| 552 | 7·34 | 98·5 | 18·50 | 94·5 | 2·8 | 7·45 |
| 550 | 7·35 | 94·5 | 18·90 | 93·2 | — | — |

as well as those made after equilibration with hydrogen, but the results indicate that the minimum pressure is not at $p_H$ 6·8 but in the region of the isoionic point, $p_H$ 7·4 ± 0·1.

In solutions containing salts, for which the isoionic point differs from the isoelectric point, the determination of the minimum osmotic pressure is difficult on account of the relatively small changes in pressure caused by alterations in the hydrogen ion concentration in the region of the isoelectric point. Experiments with 0·01 *M* phosphate buffers shown in Fig. 4 indicate a minimum value at

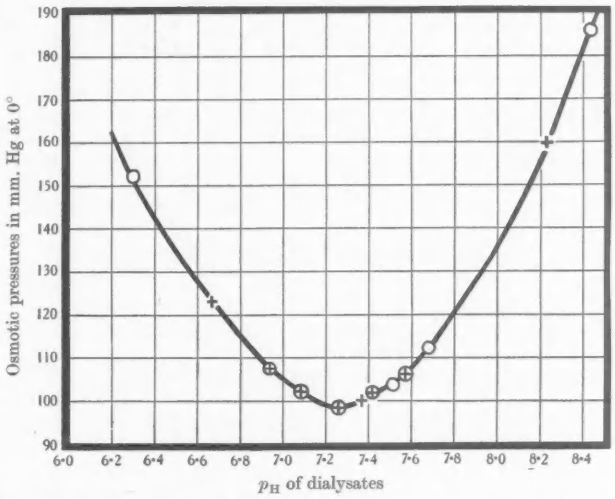



Fig. 4. Minimum osmotic pressure of sheep CO-haemoglobin, dialysates 0·01 *M* ammonium phosphates at 0°. Pressures extrapolated for $m_p = 0·004$, from data in Table IV. Circles, series 451–462; crosses 467–477; crosses enclosed in circles, mean values for both preparations.

Table XIII. *Osmotic pressures with ammonium phosphate buffers of different* $p_H$ *values.*

Sheep CO-haemoglobin. Osmotic pressures for $m_p = 0·004$, extrapolated from data in Table V. Pressures for preparation 1932, 4 asterisked.

| Ionic strength 0·01 | | Ionic strength 0·10 | | Ionic strength 0·20 | |
|---|---|---|---|---|---|
| $p_{H''}$ | $p$ | $p_{H''}$ | $p$ | $p_{H''}$ | $p$ |
| 6·33 | 173·4* | 6·00 | 118·2 | 5·84 | 115·2* |
| 6·40 | 169·2 | 6·00 | 118·6* | 5·85 | 116·7 |
| 6·82 | 130·0* | 6·57 | 109·5* | 6·43 | 115·0* |
| 6·90 | 128·2 | 6·58 | 106·2 | 6·45 | 113·2 |
| 7·13 | 113·5 | 6·88 | 107·5* | 6·77 | 110·4* |
| 7·11 | 113·5* | 6·89 | 105·8 | 6·77 | 109·1 |
| 7·42 | 100·6 | 7·36 | 107·3 | 7·27 | 110·2 |
| 7·46 | 102·2 | 7·37 | 109·1* | 7·28 | 111·4* |
| 7·48 | 102·6* | | | | |

$p_H$ 7·27 ± 0·15, a value intermediate between the isoelectric point 7·06 and the isoionic point, 7·4 ± 0·05, in the preparations referred to in Table IV. Table XIII gives provisional estimates of the pressures for solutions containing 0·004 mol.

of protein per litre of solvent, calculated from data in Table V, on the assumption that pressures and concentrations are related by the empirical formula

$$p = RT m_p + k_1 RT m_p^2.$$

In solutions of 0·01 ionic strength, where the phosphate is approximately 0·004 $M$ the minimum pressure seems to be near $p_H$ 7·4. The minimum in more concentrated buffers appears to be at more acid reactions. It may be noted that in the more alkaline solutions, the change in pressure with $p_H$ may be smaller than that calculated for the changes in the ion pressure difference by the formulae given by Adair [1929] and by Adair and Robinson [1930, 2].

9. *Relationship between the buffer value $\beta_H$ and the valence increment $\beta_n$.*

The buffer value $\beta_H$ is defined as the increase in the acid bound by one molecule (67,000 g.) of haemoglobin, divided by the increase in the $p_H$. Hastings, Van Slyke *et al.* [1924] showed that in solutions of reduced horse haemoglobin at 38°, containing 0·03, 0·05 and 0·145 equivalents of sodium ions, the buffer values are 10·0, 10·6 and 11·3 respectively. The buffer value of oxyhaemoglobin varied with the $p_H$. Human haemoglobin has a lower buffer value of 9·2 [Adair, 1925, 1]. In the case of sheep oxyhaemoglobin, the value of $\beta_H = 8$ is estimated from $CO_2$ dissociation curves [Adair *et al.*, 1929] and $\beta_H = 7{\cdot}35$–$9{\cdot}3$ from measurements made by means of the glass electrode by Terroux [1931]. In the present investigation similar measurements on sheep CO-haemoglobin made with the hydrogen electrode at 0°, shown in Fig. 6, give a buffer value of 7·7 in the region of the isoelectric point.

The estimation of buffer values from studies of membrane equilibria presents greater technical difficulties than the earlier methods, but for the purpose of this work, the method is of use as a means of comparing the buffer value and the valence increment $\beta_n$, defined as the change of valence per $p_H$ unit; $\beta_n$ cannot be determined by the earlier methods except in the special case of very dilute mixtures of protein and hydrochloric acid, referred to by Pauli and Valkó [1933].

The estimation of the valence $n_p$, from measurements of membrane potentials in buffer solutions, has been described by Adair and Adair [1934] who have given the formula

$$n_p = 0{\cdot}0085\, \mu M\, (E/C_v)_0 \qquad \text{.....(8).}$$

$M$ = mol. wt.; $\mu$ = ionic strength; $C_v$ = g. protein per 100 ml. solvent; $(E/C_v)_0$ = the limiting value of the ratio $(E/C_v)$ when $C = 0$.

In experiments in which this ratio has not been determined, approximate estimates of $n_p$ have been made by formula 9:

$$m_p n_p = [\text{free anions}]_c' - [\text{free cations}]_c' \qquad \text{.....(9).}$$

The terms in square brackets represent the sums of the corrected equivalent concentrations of free anions and free cations, estimated on the approximate assumption that concentrations of ions uncombined with proteins are the same as the "ideal" concentrations, defined by formula 6. The values of $n_p$ estimated by formula 9 are used in approximate calculations of the isoelectric point $I$ by the empirical formula $n_p = \beta_n (I - p_H)$. A similar formula $I_H = p_H + A_p/\beta_H$ is of service in estimates of the isoionic point $I_H$. The range over which $\beta_H$ and $\beta_n$ are approximately constant is shown in Figs. 5 and 6.

Table XIV gives a series of determinations of $\beta_H$ and $\beta_n$ for sheep CO-haemoglobin equilibrated with ammonium phosphate buffers of constant ionic strength, or with a constant molarity. The values of $\beta_n$ recorded are based on approximate estimates of $n_p$ made by application of formula 9, from the measurements of

membrane potential, some of which are given in Tables IV, V and X. In systems in which $E$ is below 2 millivolts, formula 9 gives the same results as formula 8,

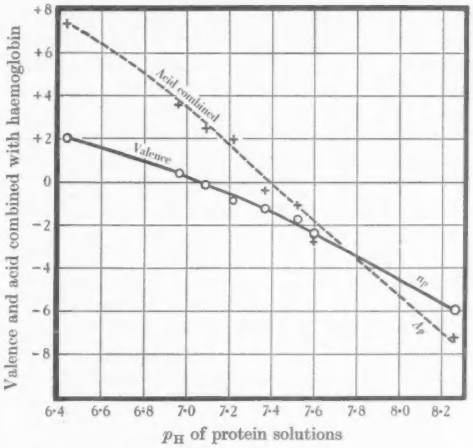



Fig. 5. Acid bound and valence of sheep CO-haemoglobin, dialysates 0·01 $M$ ammonium phosphates at 0°; calculated from Exps. 452–462, Table IV. Isoionic point at $A_p=0$.

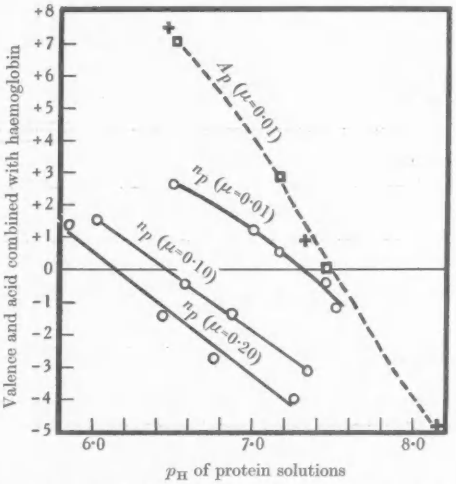



Fig. 6. Valence of sheep CO-haemoglobin, calculated from data in Table V. Dialysates ammonium phosphate buffers at 0°. Broken line, acid bound by haemoglobin; crosses, $A_p$ in mixtures containing Na, Cl and $HCO_3$; squares, $A_p$ in ammonium phosphates, calculated from data in Tables V and VI. (Crosses, calculated by method A; squares, method B, section 3).

but if $E$ exceeds 5 millivolts, there are appreciable differences, as shown by the curves given by Adair and Adair [1934]. In the experiments with buffers of ionic strength 0·01, where the potentials with 20 % solutions may exceed

9 millivolts, the value of $\beta_n$ estimated by formula 9 is 3·6, as shown in Table XIV, whereas that estimated by formula 8 is approximately 4·6. Although a number of the values in Table XIV may be subject to correction, there seems to be little doubt that $\beta_n$ is quite markedly less than $\beta_H$. This difference, discussed in Section 11, is of importance in the theoretical interpretation of the mobilities of proteins; Abramson [1933], in the absence of direct measurements, assumes that $\beta_H$ and $\beta_n$ are identical.

Table XIV. *Buffer values and valence increments of haemoglobin.*

Sheep CO-haemoglobin, dialysates phosphate buffers at 0°. Mean values of $\beta_H$ and $\beta_n$ over the same $p_H$ range. Concentrations of proteins and salts given in Tables IV, V, VI and X, except series 331–337, which is comparable with series 451–462. Asterisked figures show that $\beta_n$ and $\beta_H$ both vary with the $p_H$, and that the difference between them is greater in more acid solutions.

| Series | Notes | Isoelectric point | $p_H$ range | $\beta_n$ | $\beta_H$ |
|---|---|---|---|---|---|
| 577–580 | Ionic strength = 0·01 | 7·42 | 6·5–7·5 | 3·6 | 7·74 |
| 581–584 | ,, = 0·10 | 6·47 | 6·0–7·3 | 3·6 | — |
| 585–588 | ,, = 0·20 | 6·18 | 5·8–7·3 | 3·8 | — |
| 331–337 | Molarity = 0·01 | 7·06 | 6·7–7·5 | 4·2 | 6·9 |
| 451–462 | ,, ,, | 7·05 | 6·7–7·5 | 3·7 | 7·8 |
| 467–477 | ,, ,, | 7·00 | 6·7–7·5 | 3·9 | 7·8 |
| 467–477 | ,, ,, | 7·00 | 6·7–7·0* | 3·3* | 7·8* |
| 467–477 | ,, ,, | 7·00 | 7·0–8·0* | 4·6* | 8·4* |
| Na–$PO_4$ | ,, ,, | 7·05 | 7·0–7·6 | 3·7 | 8·6 |
| Na–Cl–$PO_4$ | ,, $PO_4$ 0·01 Cl 0·05 | 6·93 | 6·9–7·2 | 4·5 | — |

10. *Effects of salts on the isoelectric points of proteins.*

The results recorded in Fig. 7 indicate that as the salt concentration is increased, the $p_H$ of the isoelectric point of haemoglobin at 0° becomes more acid. Michaelis and Davidsohn [1912] measured the cataphoresis of haemoglobin at

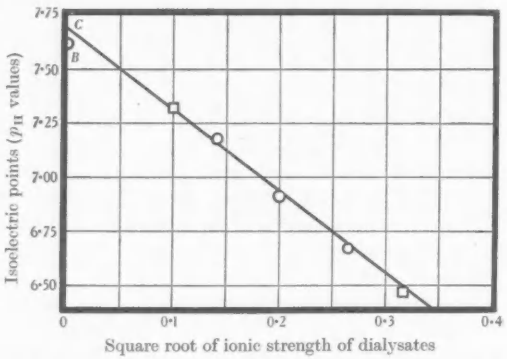



Fig. 7. Variation in isoelectric point of sheep CO-haemoglobin caused by ammonium phosphates. Circles, crystalline haemoglobin, calculated from data in Table VIII; squares, preparation 1932, 4, Table V. $C$ = isoionic point by extrapolation, $B$ = isoionic point determined from chemical analyses.

room temperature and concluded that the isoelectric point is independent of salt concentration. In view of this discrepancy, the evidence obtained by different methods as to the constancy of the isoelectric point may be briefly summarised.

The point of maximum precipitation of globulin is shifted to the acid side by the addition of neutral salts, as shown by Hardy [1905], and it may be noted that Przyłęcki [1931] regards an effect of salts on precipitation as a proof that salts change the isoelectric point, but in view of the more critical work of Hardy and of Cohn [1922], who discusses the work of Michaelis and Szent-Györgyi [1920] and of Hopkins and Savory [1911], it appears that the point of maximum precipitation depends upon a number of factors. It may be noted that in the case of haemoglobin there is a marked discrepancy between the isoelectric point and the minimum solubility, in that the isoelectric point varies by approximately 0·45 $p_H$ unit over a range of 25° [Stadie and Martin, 1924], whereas the minimum solubility of horse haemcglobin at 25° recorded by Green [1931] is the same as the value of 6·6 at 0° given by Cohn and Prentiss [1927]. Green concluded that salts alter the apparent dissociation constants and presumably the isoelectric point of haemoglobin. A minimum solubility can be obtained where haemoglobin is not isoelectric, as shown by Sørensen and Sørensen [1933], who published a curve with two minima, at $p_H$ 5·4 and at $p_H$ 6·6.

The point of minimum swelling of gelatin may be altered by the addition of salts [Kuntzel, 1929], but here again it can be shown that the evidence is indecisive. Swelling is correlated with osmotic pressure, which in the case of a protein, can be regarded as the sum of two terms, namely $p_i$, the pressure due to the diffusible ions, and $p_p$, the partial pressure of the protein [Adair, 1928; 1929]. The term $p_i$ is zero at the isoelectric point and, if $p_p$ is unaffected by hydrogen ions, minimum osmotic pressure and swelling should correspond with the isoelectric point, but if hydrogen ions alter the state of aggregation of the protein, or the forces between the particles, the observed minimum is a function of two variables and not necessarily equal to the isoelectric point.

Although the measurements of Michaelis and his colleagues showed no evidence for an effect of salts on the isoelectric point, the more accurate apparatus devised by Tiselius [1930] gave an isoelectric point of $p_H$ 4·55 for egg-albumin in acetate buffers of 0·02 ionic strength, containing 0·02 mol. of sodium acetate per litre, whereas the isoionic point is at $p_H$ 4·76. Abramson [1933] supports the view that the isoelectric point can be changed by the addition of salts. The measurement made by Tiselius is of greater importance as evidence for a change of isoelectric point than are the results obtained from observations of maximum precipitation and swelling, as the movement of protein particles in an electric field should be more directly correlated with the electric charge on the particles, but the evidence cannot be regarded as an unqualified proof, in view of the observations of Loeb [1924] and of Freundlich [1930] that the cataphoresis of air bubbles is greatly affected by the addition of electrolytes, which suggests that suspended particles are surrounded by a film of water, containing an excess of one species of ion, usually the anion, which moves with the particle. It follows that the addition of electrolytes may cause an uncharged particle to move in an electric field. The theory that a sheath of solution moves with the particle is of interest in connection with the differences between the results of Michaelis and those obtained in the present work. In measurements of membrane potentials, the diffusible ions surrounding the protein are in a state of thermodynamic equilibrium, unaffected by an external electric field.

Loeb [1924] made a series of comparisons of the cataphoric potential $\zeta$, and of the membrane potential, and concluded that salts with tri- or quadri-valent ions may reverse $\zeta$, but only bring the membrane potentials to zero. In the present investigation, where the membrane potential measurements have been more closely spaced, it is evident that there is a limited range of $p_H$ values,

shown in Fig. 2, where an increase in phosphate concentration can alter the sign of the membrane potential.

The evidence for a variation in isoelectric point with salt concentration, obtained by measurements of membrane potentials, is of interest as it seems probable that the electric charge or valence of protein ions is correlated with the membrane potentials by the simple formula 8, discussed by Adair and Adair [1934]. It is possible, however, that the results may require correction, as they are based on the approximate assumption that a saturated solution of potassium chloride annuls the liquid junction potentials. The curves in Fig. 2 show that the addition of a small constant correction of 0·3 mv. to the observed potentials tends to diminish the distance between the isoelectric points at different ionic strengths.

The hypothesis that the changes in the isoelectric point caused by salts are entirely due to the liquid junction potentials may be advanced in order to reconcile the results with the conclusion of Michaelis, but it is necessary to postulate the existence of specific effects of haemoglobin on the liquid junction potentials ranging from 1·5 to 3 mv. at different salt concentrations. In the absence of independent evidence, it seems difficult to accept this hypothesis. No evidence of the existence of marked differences between the liquid junctions potentials, due to the unequal concentrations of diffusible ions in the protein solution and the dialysate, was obtained in the comparison of the membrane potentials of haemoglobin with ammonium phosphate and with sodium phosphate buffers, shown in Fig. 3. In systems where the diffusion potentials play an important part in the observed potentials, the replacement of sodium by an ion of higher mobility, such as potassium or ammonium, may alter the potentials by several millivolts [Bjerrum and Unmack, 1929]. The experiments show that there is not much difference between the values of $dE/dp_H$ measured with sodium and with ammonium phosphates.

### 11. *Chemical significance of the difference between isoelectric and isoionic points.*

The curves in Figs. 5, 6 and 7 show that in the presence of salts, the isoelectric point of haemoglobin is on the acid side of the isoionic point, at which the protein appears to have a negative charge. The values of the isoelectric points in 0·01 *M* ammonium phosphate buffers, obtained with different preparations of haemoglobin, are not identical, but they all agree in showing a difference of about 0·4 $p_H$ unit from the isoionic points of the preparations in the same buffer mixture. The results may require corrections, referred to in Section 10, but it seems probable that the effects of salts on the isoelectric point are mainly due either to the formation of a compound between the protein and the negatively charged phosphate ions, or to the formation round the particle of a layer, containing an excess of phosphate ions. On both of these hypotheses, the protein may diminish not only the activity coefficient of the phosphate ions, a conclusion in accordance with the data in Table XV, but also the mean activity coefficient of the ammonium and phosphate ions, symbolised $f_{\pm}$, which can be investigated by chemical methods, without using cells with liquid junctions. In the case of Exp. 654, given in Table I, where the products of the corrected concentrations of the ions $NH_4^+$ and $H_2PO_4^-$ in the protein solution and in the dialysate are 0·000556 and 0·0003 respectively, the protein diminishes the mean activity coefficient of the ions by 26·5 %.

Calculations based on the data for 0·01 *M* buffers, given in Table IV, show that the protein may cause even greater diminutions in $f_{\pm}$. Similar effects are

obtained for sodium phosphates, but the effect of the protein on the mean activity coefficient of sodium chloride is smaller (approximately 10 %, in the case of Exps. E and F in Table X), in accordance with the relatively small change in the isoelectric point caused by sodium chloride. Stadie and Sunderman [1931] measured the freezing-points of mixtures of haemoglobin and salts and concluded that the protein causes no change in the mean activity coefficient, but their observations were not made under the most favourable conditions, namely a high concentration of protein and a low concentration of salts.

The discrimination between the effects of interionic attraction and of chemical combination is a difficult problem. The suggestion that proteins form compounds with heavy metals and with alkaline earths has been made by a number of investigators, referred to by Pauli and Valkó [1933], who have given an extensive list of references to papers bearing on the alternative hypothesis that proteins attract ions. The existence of compounds between proteins and the simpler ions $Na^+$, $Cl^-$ and $HCO_3^-$ has been postulated to account for the effects of salts on the solubility of proteins [Kondo and Hayashi, 1928], but in view of the lack of discrimination between combination and attraction, and the absence of quantitative data as to the composition of the supposed compounds, their existence is generally discredited.

Evidence in favour of the existence of interionic attractions and repulsions has been obtained in measurements of the osmotic pressure of haemoglobin [Adair, 1928] and of the solubility of haemoglobin [Cohn and Prentiss, 1927]. In the present investigation, certain consequences of the interionic attraction theory have been compared with the data recorded in Table XV.

Table XV. *Effect of variation in the valence of haemoglobin ions on the activities of phosphate ions.*

Sheep CO-haemoglobin, dialysates 0·01 *M* ammonium phosphates. Concentrations of proteins and of salts given in Table IV. Exps. 467, 469, 475 and 477, $p_{H'}$ measured with hydrogen electrode, remaining values calculated by Formula 1. $b$ = apparent combination in equivalents of phosphate per mol. haemoglobin. Corrected concentrations of ions in mols. per l. solvent.

| Exp. no. | $p_{H''}$ | $p_{H'}$ | $[PO_4]_c'$ ideal | $[PO_4]_c'$ observed | $b$ | $x'$ | $f'/f''_{PO_4}$ | $n_p$ |
|---|---|---|---|---|---|---|---|---|
| 467 | 6·67 | 6·71 | 0·01164 | 0·02760 | 4·9 | 0·26 | 0·42 | +1·0 |
| 469 | 6·94 | 6·94 | 0·01018 | 0·02177 | 4·1 | 0·45 | 0·47 | +0·2 |
| 471 | 7·09 | 7·07 | 0·00930 | 0·01913 | 3·6 | 0·47 | 0·49 | −0·4 |
| 473 | 7·26 | 7·22 | 0·00880 | 0·01710 | 3·0 | 0·50 | 0·51 | −0·8 |
| 474 | 7·37 | 7·32 | 0·00838 | 0·01490 | 2·7 | 0·63 | 0·56 | −1·1 |
| 475 | 7·40 | 7·32 | 0·00806 | 0·01429 | 2·4 | 0·64 | 0·56 | −1·3 |
| 476 | 7·54 | 7·47 | 0·00748 | 0·01275 | 2·2 | 0·76 | 0·59 | −2·0 |
| 477 | 8·23 | 8·09 | 0·00465 | 0·00640 | 0·8 | 0·92 | 0·73 | −5·0 |

In the first place, an increase in the valence of the protein, positive or negative, increases the ionic strength of the solution, and according to the theory of Debye and Hückel [1923] the activity coefficient of the ions is reduced. The experiments recorded in Table XV show that over the range of valences from +1 to −5, protein causes the smallest change in $f'/f''_{PO_4}$ not at $n_p=0$ but at $n_p=-5$. In the second place, bivalent ions are affected more strongly than univalent ions, according to the theory, but an increase in $x'$, the fraction of the total phosphate in the bivalent form, is correlated with an increase rather than a diminution in $f'/f''_{PO_4}$. In the third place, evidence for and against the existence of compounds can be obtained by examining the equilibrium of crystals and mother-liquor, recorded in Table XI; it will be noted that there is an excess of

phosphate in the solid phase, comparable with that found for protein in solution, and in addition, there is an excess of ammonia. The presence of ammonia and phosphate in the solid phase might be anticipated on the assumption that chemical compounds are formed. The results of the three tests are in favour of combination rather than attraction.

The term $b$ in Table XV represents the "apparent combination" or the sum of the effects of attraction and combination in equivalents of phosphoric acid per mol. of haemoglobin; it may be observed that the increase in $b$ with increasing acidity accounts quantitatively for the difference between $\beta_H$ and $\beta_n$ referred to above.

In planning further tests of the theory, it must be remembered that at the isoionic point, the addition of salts gives a negative charge to the protein, as shown by the experiments made in this work, although the measurements of Hastings, Van Slyke *et al.* [1924] and of Sørensen *et al.* [1927] show that there is no change in $p_H$ on the addition of salts to isoionic protein.

These conditions are satisfied by the reaction in formula 10, suggested by Adair and Adair [1934],

$$^{+}Pr^{-} + Na^{+} + Cl^{-} = [Cl\ Pr]^{-} + Na^{+} \qquad \text{.....(10).}$$

The symbol $^{+}Pr^{-}$ represents a pair of groups with a positive and negative charge, existing on the protein molecule in an aqueous solution. The presence of such groups is highly probable, since Bjerrum [1924] has found that in neutral solutions amino-acids are represented by the zwitterion formula $^{+}H_3N.R.COO^{-}$ rather than by $NH_2R.COOH$. In proteins of molecular weight 67,000, the number of groups capable of reacting with acids or bases may exceed 200, but for the sake of simplicity, only the pair represented by the formula $^{+}Pr^{-}$ may be considered. In pure water, this group is both isoionic and isoelectric. The protein can acquire a negative charge by the loss of a hydrogen ion, which takes place when alkalis are added, but this reaction cannot account for the negative charge caused by the addition of neutral salts at the isoionic point, where the protein neither gains nor loses hydrogen ions. In the reaction suggested in formula 10, the net negative charge is attributed to the loss of a positive charge by the zwitterion, due to the formation of un-ionised protein chloride $[Cl\ Pr]^{-}$. On crystallisation, the double salt Cl.Pr.Na is obtained. On the addition of a sufficient amount of acid, a negative group of the type $R.COO^{-}$ is neutralised by a hydrogen ion, and the isoelectric protein Cl.Pr.H is obtained.

$$[Cl\ Pr]^{-} + Na^{+} + HCl = [Cl.Pr.H] + Na^{+} + Cl^{-} \qquad \text{.....(11).}$$

The crystals of isoelectric protein should contain less base, in accordance with the experiments described above.

In the case of proteins other than sheep CO-haemoglobin, very little evidence is available, since comparatively few measurements of isoionic and isoelectric points have been made on the same preparation, with the exception of the work of Tiselius [1930], referred to above, on egg-albumin. Indirect evidence for a difference between isoelectric and isoionic points has been obtained in the case of serum [Hastings *et al.*, 1927], haemoglobin [Adair, 1928] and serum-albumin, which were shown to cause a marked diminution of the activities of chlorides and other anions, and relatively little change in the activities of cations [Adair and Adair, 1934]. On the other hand, Hitchcock [1931] found no difference between the isoionic and the isoelectric points of gelatin in acetate buffers.

SUMMARY.

The possibility of determining the isoelectric point from measurements of membrane potentials has been investigated. It seems that the most accurate results are obtained under conditions where concentrated solutions of protein are equilibrated with dilute buffer solutions at 0°.

In order to facilitate comparisons of the isoelectric point with the isoionic point, at which the protein neither gives off nor takes up hydrogen ions, methods have been developed for the estimation of the isoionic point from the distribution of salts across membranes.

A study has been made of methods for the preparation of sheep CO-haemoglobin, a protein which is highly soluble at the isoelectric point. Slight alterations in the protein may take place on crystallisation, and different preparations are not always identical, but comparative results can be obtained on a given preparation.

At 0°, the isoionic point of crystallised haemoglobin was at $p_H$ 7·6. Uncrystallised protein gave lower values of $7{\cdot}45 \pm 0{\cdot}1$. In ammonium phosphate buffers, of ionic strengths 0·02, 0·04 and 0·07, the isoelectric points of the crystallised protein were 7·16, 6·91 and 6·70 respectively. Seven preparations of uncrystallised material, with buffers of ionic strength 0·02, gave an isoelectric point $7{\cdot}06 \pm 0{\cdot}01$. The isoelectric points become more acid as the salt concentration is increased; the effects of increasing concentrations of chlorides are less than those of phosphates.

There are slight but measurable differences between the results obtained with sodium and with ammonium phosphate buffers.

In sodium as in ammonium phosphate buffers, the increase in the valence of the protein correlated with increasing hydrogen ion concentration is smaller than the increase in the acid bound by the protein.

The minimum of osmotic pressure appears to be at $p_H$ values between the isoionic and isoelectric points.

The distribution of ammonia and of phosphoric acid between protein crystals, protein solution and dialysate has been investigated.

Experiments have been made to test the hypotheses that the proteins combine with ions, or that they attract the ions in their neighbourhood, and it is suggested that the effects of salts on the isoelectric point, and the effects of proteins on the activities of diffusible ions may be due to a reaction of the type $^{+}Pr^{-} + Na^{+} + Cl^{-} = [\overset{\frown}{Cl}\ Pr]^{-} + Na^{+}$, where $^{+}Pr^{-}$ represents a zwitterion.

REFERENCES.

Abramson (1933). *J. Gen. Physiol.* **16**, 593.
Adair (1925, 1). *J. Biol. Chem.* **63**, 493.
—— (1925, 2). *Proc. Roy. Soc. Lond.* A **108**, 627.
—— (1928). *Proc. Roy. Soc. Lond.* A **120**, 537.
—— (1929). *Proc. Roy. Soc. Lond.* A **126**, 16.
—— and Adair (1934). *Biochem. J.* **28**, 199.
—— Cordero and Shen (1929). *J. Physiol.* **67**, 288.
—— and Keys (1934). *J. Physiol.* **81**, 162.
—— and Robinson (1930, 1). *Biochem. J.* **24**, 993.
—— —— (1930, 2). *Biochem. J.* **24**, 1864.
Barcroft (1928). The respiratory function of the blood. Part 2. Haemoglobin. (Cambridge.)
Bjerrum (1924). *Z. physikal. Chem.* **110**, 656.
—— and Unmack (1929). *Kgl. Danske. Vid. Selsk. Mat. Fys. Med.* (9), 1.

Bolam (1932). The Donnan equilibrium. (London.)
Clark (1928). The determination of hydrogen ions. (Baltimore.)
Cohn (1922). *J. Gen. Physiol.* **4**, 697.
—— and Prentiss (1927). *J. Gen. Physiol.* **8**, 619.
Debye and Hückel (1923). *Physik. Z.* **24**, 185.
Donnan and Allmand (1914). *J. Chem. Soc.* **105**, 1941.
Errera and Hirschberg (1933). *Biochem. J.* **27**, 764.
Ferry (1923). *J. Biol. Chem.* **57**, 819.
Freundlich (1930). Kapillar Chemie. 1. (Leipzig.)
Geiger (1931). *Proc. Roy. Soc. Lond.* B **107**, 368.
Green (1931). *J. Biol. Chem.* **93**, 517.
Hardy (1899). *J. Physiol.* **24**, 288.
—— (1905). *J. Physiol.* **33**, 251.
Hastings, Van Slyke, Neill, Heidelberger and Harington (1924). *J. Biol. Chem.* **60**, 89.
—— Salvesen, Sendroy and Van Slyke (1927). *J. Gen. Physiol.* **8**, 701.
—— Sendroy, Murray and Heidelberger (1924). *J. Biol. Chem.* **61**, 317.
Henriques (1929). *Ergebn. Physiol.* **28**, 625.
Hitchcock (1931). *J. Gen. Physiol.* **14**, 685.
Hopkins and Savory (1911). *J. Physiol.* **42**, 189.
Kondo and Hayashi (1928). *Memoirs of the College of Agriculture*, Kyoto, No. 5, 32.
Kuntzel (1929). *Biochem. Z.* **209**, 326.
Loeb (1924). Proteins and the theory of colloidal behaviour. (New York.)
Michaelis and Davidsohn (1912). *Biochem. Z.* **41**, 102.
—— —— Szent-Györgyi (1920). *Biochem. Z.* **103**, 178.
Pauli and Valkó (1933). Kolloidchemie der Eiweisskorper. (Dresden.)
Pedersen (1933). *Kolloid. Z.* **63**, 268.
Przyłęcki (1931). *Biochem. J.* **25**, 713.
Roche, Roche, Adair and Adair (1932). *Biochem. Z.* **26**, 1811.
Sørensen (1917). *Compt. Rend. Lab. Carlsberg*, **13**, No. 5.
—— Linderstrøm-Lang and Lund (1927). *J. Gen. Physiol.* **8**, 543.
—— and Sørensen (1933). *Compt. Rend. Lab. Carlsberg*, **19**, 11.
Stadie and Martin (1924). *J. Biol. Chem.* **60**, 191.
—— —— Sunderman (1931). *J. Biol. Chem.* **91**, 227.
Svedberg (1930). *Kolloid. Z.* **51**, 10.
—— (1933). *J. Biol. Chem.* **103**, 311.
Taylor (1924). *Proc. Roy. Soc. Lond.* B **96**, 383.
Terroux (1931). *J. Physiol.* **71**, 323.
Tiselius (1930). *Nova Acta Reg. Soc. Sci. Upsaliensis*, Ser. 4, **7**, No. 4.
Van Slyke, Hastings, Heidelberger and Neill (1922). *J. Biol. Chem.* **54**, 481.
—— and Hiller (1928). *J. Biol. Chem.* **78**, 807.
Walker and Williamson (1920). *J. Biol. Chem.* **41**, 75.

# CLXXII. GROWTH AND VITAMIN A DEFICIENCY[1].

By JOHN BOYD ORR and MARION BROCK RICHARDS.

*From the Rowett Research Institute, Aberdeen.*

(*Received May 5th, 1934.*)

The idea that vitamin A has a specific effect in promoting growth has been prevalent since the earliest days of vitamin work. Thus when accumulating evidence indicated that a fat-soluble accessory factor was necessary for satisfactory growth in rats, Hopkins [1912] suggested that the missing factor was one "concerned solely with growth," and Osborne and Mendel [1913] sought to discover the factor which is "peculiarly essential for growth." Even after the fat-soluble factor had been differentiated into two components, and it had been shown that absence of the antirachitic component D might also be a limiting factor in growth [Steenbock and Nelson, 1923], the tendency still persisted to regard component A as the "growth-promoting vitamin," and references to its activity in this respect have abounded in the literature up to the present day.

Although the early experimental evidence may have given some justification for assuming that the fat-soluble factor had a specific effect on growth, further research on the rôle of vitamin A in nutrition has indicated that the pathological conditions arising from deficiency of this vitamin are of more importance than any apparent cessation of growth that may occur. A few workers have realised the significance of these observations, and have protested against the continued use of the term "growth vitamin" for vitamin A [*e.g.* Steenbock *et al.*, 1924–25; Green and Mellanby, 1928], but the idea is still widely accepted. Thus, in the monograph on the "Vitamins" recently published by the Medical Research Council [1932], it is stated that "the biological estimation of vitamin A is generally based on its growth-promoting property," that "in a curative test, the animals are allowed to exhaust their reserves of vitamin A, as indicated by cessation of growth," and that "the timely administration of adequate supplies of vitamin A is soon followed by a resumption of growth." Still more recently we find papers published in Germany and France, bearing in their titles, with reference to vitamin A, the phrases "Das Vitamin des Wachstums" and "Le Facteur de Croissance" [Kuhn and Brockman, 1933; Busson, 1933].

Although there is abundant evidence of the fall in weight which eventually occurs in the absence of this vitamin, no proof has been given of the generally accepted assumption that this loss in weight indicates cessation of growth. The weight of an animal is dependent partly on the weight of its skeleton, but to a large extent also on the weight of its organs, and on the amount of muscle and fat which cover the skeletal framework. The weight is thus very readily affected by variations in the health and state of nutrition of the animal. On the other hand, size, stature or length is determined almost entirely by the amount of skeletal development. It is a commonplace clinical observation that a pathological condition may be accompanied by loss of weight, and further, that in

[1] A preliminary outline of this work has already been published [Orr and Richards, 1934].

spite of loss in weight a child during a long illness may increase considerably in height. It is thus obvious that failure to increase in weight is not necessarily synonymous with failure to "grow." It cannot therefore be claimed that loss of weight indicates cessation of growth unless it can be demonstrated that the animal has ceased to increase in length as well as in weight, and that skeletal growth has ceased. The investigations here recorded were therefore undertaken to ascertain, by measurements of body length in the live animal and of bone length *post mortem*, whether growth as determined by increase in length of the skeleton, and in particular of the long bones, does in fact cease in vitamin A deficiency when the animal ceases to increase in weight.

## Experimental.

The *basal diet* used (Diet L) was that recommended by Sampson *et al.* [1932], except that a small amount of potassium iodide was added, and vitamin D was supplied as radiostol instead of by irradiation of cotton-seed oil. This diet, which contains a high percentage of yeast (20 % of the dry substances), proved eminently satisfactory. It seemed to provide a sufficiency of all dietary factors other than vitamin A, since rats fed on the diet, supplemented by an adequate dose of vitamin A concentrate, showed normal weight curves throughout the period of 12–13 weeks covered by the experiment. In certain cases our tests involved the addition of a supplement of dried separated milk to the basal diet. Twelve rats on this diet (L+M) served as negative controls, the absence of vitamin A from both the basal diet and the added dried milk being indicated by the weight curves, which were typical of those obtained on diets containing no vitamin A.

### 1. *Live measurements.*

(*a*) *Measurements of tail growth in rats receiving a small dose of vitamin A.* Some preliminary tests were carried out in which increase in the length of the tail was used as an indication of growth. Measurements of the length of the tail were made twice weekly on a series of rats which had received from weaning a small dose of vitamin A in addition to the basal diet L. The supplement given was 0·075 g. of "fortified milk," *i.e.* dried separated milk to which had been added before evaporation a vitamin A concentrate. The small amount of

Table I. *Growth of tail in rats receiving an inadequate dose of vitamin A.*

Each measurement is the average of three successive determinations made on dates 3–4 days apart, and each weight given is the average of the weights on the dates of measurement.

| | Length of tail in cm. | | | | Weight in g. | | | |
|---|---|---|---|---|---|---|---|---|
| Period ... | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Rat 2581 | 16·5 | 16·7 | 16·8 | — | 151 | 143 | 123 | — |
| 2582 | 16·8 | 16·9 | 17·0 | 17·2 | 156 | 144 | 147 | 146 |
| 2612 | — | *14·4 | 14·6 | 14·8 | — | 139 | 139 | 136 |
| 2636 | 15·5 | 15·9 | 15·9 | 16·3 | 136 | 137 | 131 | 133 |
| 2638 | *12·5 | 13·4 | 13·5 | 13·7 | 154 | 165 | 159 | 148 |
| 2640 | 15·6 | 15·9 | 16·5 | 16·8 | 140 | 156 | 158 | 138 |
| 2585 | 15·1 | 15·4 | 15·9 | 16·1 | 108 | 112 | 109 | 106 |
| 2596 | 14·3 | 14·9 | 15·3 | — | 116 | 110 | 106 | — |
| 2603 | 13·9 | 14·6 | 15·0 | 15·0 | 115 | 120 | 121 | 117 |
| 2607 | 14·0 | 14·4 | 14·5 | — | 85 | 87 | 71 | — |
| 2634 | 15·1 | 15·6 | 15·9 | 16·3 | 104 | 104 | 103 | 105 |
| 2635 | 14·1 | 15·0 | 15·3 | 15·6 | 110 | 114 | 118 | 117 |

* Tip of tail missing.

vitamin A potency retained in the evaporation of the "fortified milk" sufficed to prolong the lives of the rats very considerably in comparison with the negative controls, and the tail measurements could thus be continued for some time. The tail was measured from base to tip by holding it firmly, without actual stretching, along the edge of a measuring rule, 2 or 3 readings being taken. The results are shown in Table I, which gives measurements and weights for periods of approximately 10 days each. Although the degree of accuracy obtainable by this method is not high, comparison of the successive weights from period to period with the measurements of tail length for the corresponding periods shows that there is in each case a distinct increase in tail length after increase in weight has ceased.

(*b*) *Measurements of body length and tail length in rats receiving no vitamin A.* In the second series of live measurements comparison was made between animals receiving no vitamin A (Diet L or Diet L+M) and positive controls which received Diet L supplemented by an ample dose of vitamin A concentrate (Diet L+A). The dose given was 4 mg. per rat daily, the amount being based on the blue unit value of the concentrate at the beginning of the experiment (700 C.P. units). The rats available, which were of the black-and-white (Lister) strain, were divided as far as possible into sets of three litter-mates of the same sex, each rat of a set receiving one of the three diets L, L+M or L+A, after weaning at the age of 23 days.

In order to increase the accuracy of the measurements, the animals were anaesthetised once a week with a mixture of nitrous oxide and oxygen, and measurements were made both of tail-length, in the manner already described, and of the total length from nose to tail. The total length was measured by means of a board with an upright end-piece against which the nose of the rat was held. The body and tail were straightened out, and the position of the tip of the tail was marked on the graph paper with which the board was covered, two or three readings being taken. It is to be noted that the length of the body alone is not given accurately by the difference between the two sets of measurements thus obtained, since in measuring the total length the tail was more relaxed than when it was held along the rule for direct measurement. The data have therefore been used to construct for each animal two independent growth curves[1] based on length of tail, and total length of body + tail, as measured under these conditions. A number of these growth curves, along with the weight curves[1] of the rats in question, are shown in Fig. 1. It will be seen that while there is no very great increase in length during the period of flattening of the weight curve, there is at the same time no indication of any "cessation of growth." The curves of tail-length and total length are simply of the nature of continuous slow growth curves. It is to be noted, moreover, that by the time the weight curves of the deficient rats are beginning to fall off, the growth curves, though not the weight curves, of the positive controls have already begun to flatten considerably, *e.g.* Rats 3220 and 3231 (Fig. 1 *b*). Further, if the weight of the vitamin A-deficient rat has been well maintained until shortly before death, *e.g.* Rats 3257, 3378 and 3314 (Fig. 1 *a*), it will be seen that the growth curves are practically identical with those of the positive controls. Thus these animals have reached a moribund condition through vitamin A deficiency before there has been any marked slackening in the rate of growth.

The curves for Rat 3289 (Fig. 1 *b*) which received an inadequate dose of vitamin A after a "depletion" period, show that considerable growth took place for a period of 25 days after the weight began to fall, while the curves for

[1] These are graphs of the observations, not statistically fitted curves.

Rat 3265, which suffered severely from the anaesthetising, provide an example of retardation of growth occurring in the presence of ample vitamin A. (It is to be noted that this retardation of growth was accompanied by a very pronounced reduction in food consumption, and that the subsequent rise in both weight and

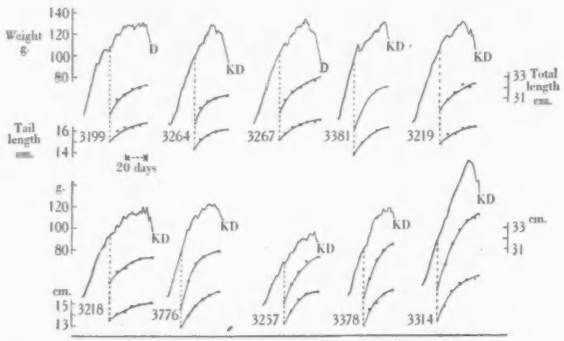



Fig. 1*a*. Weight and growth curves for rats receiving no vitamin A. In each set of 3 curves, the top, middle and bottom curves represent weight, total length and tail length respectively.

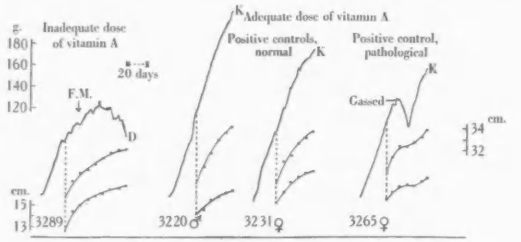



Fig. 1*b*. Weight and growth curves for rats receiving inadequate or adequate doses of vitamin A. Note the pathological retardation of growth in Rat 3265, in spite of the presence of ample vitamin A.

In Figures 1, 3 and 4, D = died; KD = killed, moribund; K = killed, not moribund.

growth curves coincided with a return to normal food intake.) It is clear therefore that it is not the presence or absence of vitamin A in itself that is the determining factor in the continuation of growth as estimated by increase in length. Growth in this sense does not cease when vitamin A is absent, or present in inadequate amounts, and on the other hand the rate of growth may be very considerably retarded in presence of adequate vitamin A, if the animal is in a pathological condition from some other cause.

### 2. *Skeletal growth.*

As it is obviously impossible to measure bones accurately during the life of the animal, an indirect method based on *post mortem* measurements was adopted to ascertain whether the bones continued to grow in length after increase in weight ceased. If weight be a measure of growth, then we should expect to find a relationship between the length of the bones and weight, and if growth ceases in vitamin A-deficient rats when increase in weight has ceased, then the bones

Table II. *Weights and bone lengths of positive controls:* i.e. *rats receiving basal diet+adequate vitamin A.*

| No. of rat | Sex | Wt. when killed (g.) | Age when killed (days) | Lengths of bones: Femur cm. | Tibia cm. | Humerus cm. |
|---|---|---|---|---|---|---|
| 3461 | ♀ | 81 | 44 | 2·38 | 2·85 | 1·91 |
| 3503 | ♀ | 81 | 44 | 2·32 | 2·73 | 1·86 |
| 3462 | ♂ | 96 | 51 | 2·59 | 2·98 | 2·02 |
| 3502 | ♀ | 98 | 44 | 2·45 | 2·87 | 1·93 |
| 3512 | ♀ | 100 | 44 | 2·50 | 2·92 | 2·00 |
| 3275 | ♀ | 103 | 66 | 2·61 | 3·04 | 2·02 |
| 3445 | ♀ | 107 | 51 | 2·60 | 3·00 | 2·06 |
| 3494 | ♀ | 109 | 44 | 2·50 | 2·88 | 1·94 |
| 3453 | ♀ | 111 | 44 | 2·53 | 2·98 | 1·98 |
| 3451 | ♂ | 113 | 51 | 2·69 | 3·10 | 2·10 |
| 3499 | ♂ | 115 | 44 | 2·58 | 2·97 | 2·03 |
| 3510 | ♂ | 117 | 44 | 2·67 | 3·06 | 2·09 |
| 3174 | ♀ | 118 | 74 | 2·69 | 3·12 | 2·09 |
| 3410 | ♀ | 119 | 58 | 2·71 | 3·15 | 2·10 |
| 3507 | ♂ | 120 | 44 | 2·59 | 3·00 | 2·04 |
| 3394 | ♀ | 120 | 58 | 2·68 | 3·05 | 2·11 |
| 3274 | ♀ | 121 | 73 | 2·67 | 3·09 | 2·05 |
| 3438 | ♀ | 126 | 51 | 2·73 | 3·09 | 2·16 |
| 3326 | ♀ | 132 | 57 | 2·78 | 3·20 | 2·18 |
| 3395 | ♀ | 133 | 58 | 2·79 | 3·19 | 2·19 |
| 3428 | ♀ | 135 | 51 | 2·77 | 3·16 | 2·17 |
| 3416 | ♂ | 135 | 58 | 2·79 | 3·24 | 2·13 |
| 3329 | ♂ | 137 | 59 | 2·74 | 3·17 | 2·10 |
| 3284 | ♂ | 137 | 74 | 2·78 | 3·18 | 2·12 |
| 3362 | ♂ | 141 | 65 | 2·98 | 3·39 | 2·32 |
| 3161 | ♀ | 145 | 73 | 2·79 | 3·24 | 2·21 |
| 3437 | ♀ | 148 | 58 | 2·81 | 3·27 | 2·21 |
| 3309 | ♀ | 148 | 67 | 2·82 | 3·18 | 2·17 |
| 3285 | ♂ | 148 | 74 | 2·94 | 3·41 | 2·22 |
| 3454 | ♂ | 149 | 50 | 2·89 | 3·30 | 2·26 |
| 3429 | ♂ | 149 | 58 | 2·91 | 3·30 | 2·29 |
| 3255 | ♀ | 152 | 75 | 2·79 | 3·20 | 2·16 |
| 3266 | ♀ | 155 | 80 | 2·82 | 3·26 | 2·19 |
| 3443 | ♂ | 155 | 51 | 2·91 | 3·32 | 2·22 |
| 3312 | ♀ | 158 | 73 | 3·00 | 3·39 | 2·32 |
| 3170 | ♀ | 161 | 87 | 2·78 | 3·18 | 2·18 |
| 3169 | ♂ | 164 | 81 | 2·91 | 3·32 | 2·25 |
| 3153 | ♀ | 164 | 89 | 2·97 | 3·39 | 2·28 |
| 3176 | ♂ | 165 | 79 | 2·88 | 3·29 | 2·24 |
| 3377 | ♂ | 166 | 75 | 2·98 | 3·40 | 2·28 |
| 3431 | ♂ | 167 | 58 | 2·93 | 3·35 | 2·28 |
| 3231 | ♂ | 170 | 85 | 2·92 | 3·31 | 2·26 |
| 3291 | ♂ | 172 | 65 | 2·88 | 3·35 | 2·19 |
| 3200 | ♀ | 172 | 87 | 3·04 | 3·46 | 2·36 |
| 3277 | ♂ | 178 | 82 | 3·05 | 3·50 | 2·32 |
| 3142 | ♂ | 178 | 103 | 2·96 | 3·41 | 2·36 |
| 3216 | ♂ | 198 | 83 | 3·06 | 3·51 | 2·35 |
| 3177 | ♂ | 199 | 93 | 3·18 | 3·59 | 2·44 |
| 3236 | ♂ | 201 | 64 | 3·05 | 3·48 | 2·30 |
| 3212 | ♂ | 206 | 86 | 3·24 | 3·69 | 2·49 |
| 3220 | ♂ | 208 | 89 | 3·12 | 3·50 | 2·34 |
| 3379 | ♂ | 217 | 77 | 3·30 | 3·71 | 2·52 |
| 3210 | ♂ | 219 | 86 | 3·28 | 3·75 | 2·49 |
| 3331 | ♂ | 221 | 75 | 3·13 | 3·54 | 2·40 |
| 3147 | ♂ | 230 | 80 | 3·28 | 3·64 | 2·51 |
| 3224 | ♂ | 232 | 87 | 3·20 | 3·64 | 2·46 |
| 3225 | ♂ | 241 | 87 | 3·23 | 3·66 | 2·47 |
| 3173 | ♂ | 247 | 94 | 3·22 | 3·64 | 2·50 |
| 3204 | ♂ | 248 | 84 | 3·29 | 3·72 | 2·53 |
| 3366 | ♂ | 252 | 109 | 3·50 | 3·90 | 2·70 |

should correspond in length with the highest weight attained by the rat, *i.e.* they should be approximately the same as might be expected in a normal rat of that weight. On the other hand, if the bones continue to grow after the animal has ceased to increase in weight, the ratio of the length of the bones to the maximum weight of the animal will be greater for the rats deprived of vitamin A than for those on adequate diet.

*Bone measurements.* The animals used for bone measurements comprised not only those on which live measurements had been carried out, but also many other similar sets of 3 rats on the diets L, L+M and L+A respectively. A number of the sets were killed off after 3, 4, 5 and 6 weeks on the various diets. In the remaining sets each of the rats on deficient diet was allowed to become moribund before being killed, and the positive control of the set was killed along with the second of the deficient rats. A *post mortem* examination was carried out on all the rats. Thereafter the right femur, tibia and humerus were dissected and cleaned, and the lengths of the fresh bones were measured by means of sliding callipers with vernier scale, the results being expressed in centimetres to two decimal places.

*Correlation between bone length and weight in normal animals.* For the determination of the relationship between bone length and body weight in normal animals under the experimental conditions, data were available for 60 positive controls representing 29 litters. These data are presented in Table II, any controls in which pathological condition had caused abnormalities in the weight curve being omitted. It will be observed that considerable variations occurred in the rates of growth of these controls. This is frequently the case with rats bred specially for vitamin work, since the common practice of restricting the vitamin content of the mother's diet with the object of "reducing the reserve stores of vitamin" in the young, undoubtedly tends to produce subnormal and variable litters. Since the vitamin A-deficient rats are in general growing at a slower rate than the controls, it is essential, before making comparisons of bone lengths in the two series, to ascertain whether in the normal series the weight or the age of the animal has the greater influence in determining bone length.

A statistical analysis of the data in Table II showed that the correlation between weight and length of bone is high, the correlation coefficients ($r$) for femur, tibia and humerus being 0·95, 0·96 and 0·93 respectively (Table III).

Table III.

| Characters | Correlation ratios | | Correlation coefficients | |
|---|---|---|---|---|
| | Positive controls | V.A.D. rats | Positive controls | V.A.D. rats |
| T W | 0·952 | 0·951 | 0·950 | 0·948 |
| F W | 0·968 | 0·968 | 0·958 | 0·966 |
| H W | 0·938 | 0·951 | 0·933 | 0·948 |

W = Body weight T = Tibia F = Femur H = Humerus V.A.D. = Vitamin A-deficient

Length of bone and age are less highly correlated. In the case of the tibia "$r$" was found to be 0·95 for bone length and weight, as against $r = 0{\cdot}80$ for bone length and age. The graphs in Fig. 2, in which bone length has been plotted for the 60 positive controls both against weight and age of rat, indicate that for the femur and humerus also the correlation between bone length and weight is much closer than that between bone length and age.

The best fitting curves of bone lengths on weights[1] were then determined in order to find the theoretical weights for each length of bone in the control series. These theoretical weights were used as standards with which the data for the

[1] See next page for equations for the curves.

deficient animals were compared, in order to determine whether in animals suffering from avitaminosis-A the normal relationship between bone length and

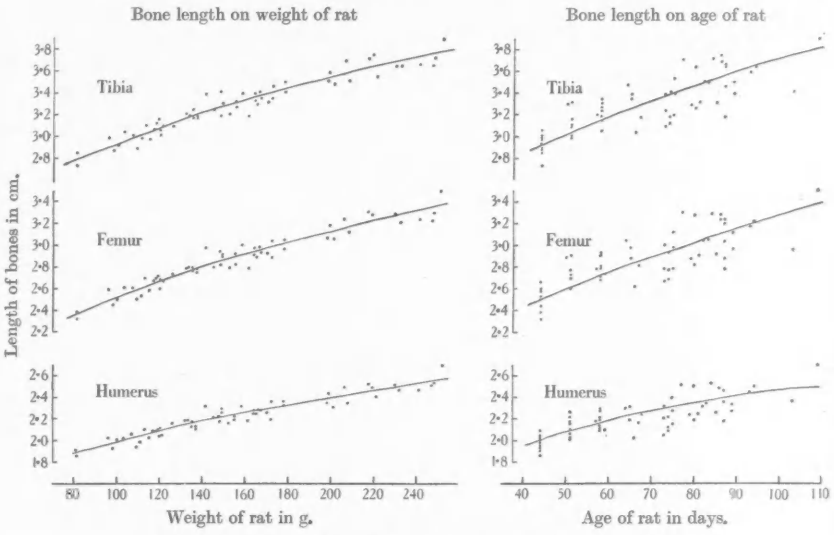



Fig. 2. Normal rats. Graphs showing closer correlation of bone length and weight than bone length and age.

body weight had been maintained. The standard curves are shown in Fig. 3, and the corresponding numerical data in Table IV.

1. The equations for the curves are:

$$\text{Tibia parabola:} \quad y = 89{\cdot}554 - 120{\cdot}528x + 42{\cdot}720x^2,$$
$$\text{Femur parabola:} \quad y = 235{\cdot}025 - 224{\cdot}247x + 68{\cdot}200x^2,$$
$$\text{Humerus parabola:} \quad y = -141{\cdot}697 + 35{\cdot}400x + 44{\cdot}270x^2,$$

where $y$=predicted weight of rats in g.
and $x$=length of bone in cm.

Table IV.

| Tibia length cm. | Weight Control g. | Weight V.A.D. g. | Femur length cm. | Weight Control g. | Weight V.A.D. g. | Humerus length cm. | Weight Control g. | Weight V.A.D. g. |
|---|---|---|---|---|---|---|---|---|
| 2·75 | 81·17 | — | 2·35 | 84·68 | — | 1·85 | 75·31 | — |
| 2·85 | 93·04 | 85·74 | 2·45 | 94·99 | 85·24 | 1·95 | 95·67 | 88·82 |
| 2·95 | 105·77 | 90·69 | 2·55 | 106·67 | 93·61 | 2·05 | 116·92 | 103·46 |
| 3·05 | 119·35 | 97·59 | 2·65 | 119·70 | 102·15 | 2·15 | 139·05 | 117·41 |
| 3·15 | 133·78 | 106·44 | 2·75 | 134·11 | 110·88 | 2·25 | 162·07 | 130·67 |
| 3·25 | 149·07 | 117·23 | 2·85 | 149·88 | 119·79 | 2·35 | 185·97 | 143·23 |
| 3·35 | 165·21 | 129·97 | 2·95 | 167·41 | 128·88 | 2·45 | 210·76 | 155·11 |
| 3·45 | 182·21 | 144·66 | 3·05 | 185·50 | 138·15 | 2·55 | 236·44 | 166·29 |
| 3·55 | 200·06 | — | 3·15 | 205·36 | 147·61 | 2·65 | 263·00 | 176·78 |
| 3·65 | 218·76 | — | 3·25 | 226·58 | — | — | — | — |
| 3·75 | 238·32 | — | 3·35 | 249·17 | — | — | — | — |
| 3·85 | 258·74 | — | 3·45 | 273·12 | — | — | — | — |

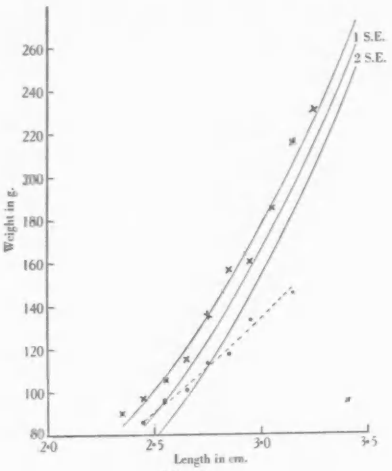



Fig. 3*a*.

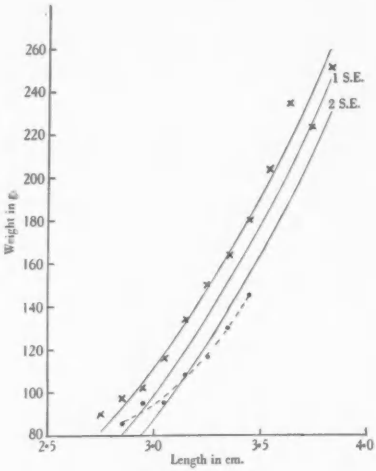



Fig. 3*b*.

Fig. 3*a*. Weight of rat on length of femur. —— Control rats. ---- V.A.D. rats.

Fig. 3*b*. Weight of rat on length of tibia. —— Control rats. ---- V.A.D. rats.
1 S.E. = Curve of standard error. 2 S.E. = Curve of twice standard error.

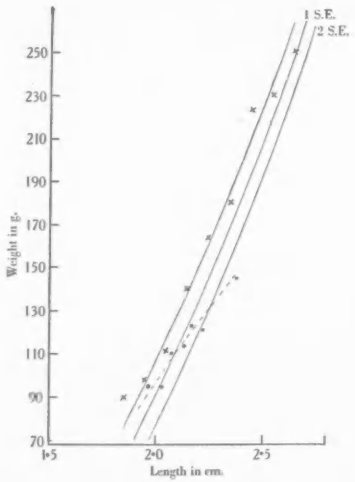



Fig. 3*c*. Weight of rat on length of humerus. —— Control rats. ---- V.A.D. rats.
1 S.E. = curve of standard error. 2 S.E. = Curve of twice standard error.

*Application to vitamin A-deficient rats.* The method of using the standard curves may be illustrated by two examples. For rat 3201 ♀ (Diet L+M), the lengths of femur, tibia and humerus were 2·81, 3·24 and 2·18 cm. respectively. From the standard curves the predicted weights for these bone lengths should be 143·3, 147·3 and 146 g.; *i.e.* rat 3201 had the skeletal development of a rat of 145·5 g. in the control series, while the highest weight attained was only 117 g. Similarly rat 3203 ♂ (Diet L), had bone lengths of 3·12, 3·50 and 2·36 cm., which should correspond with weights of 199, 190·8 and 188 g. Thus this animal, for which the highest weight recorded was 144 g., had the bone lengths of a rat of 192·6 g. By interpolating these values in the weight graphs, we obtain a graphical representation of bone growth as contrasted with weight in the deficient rats.

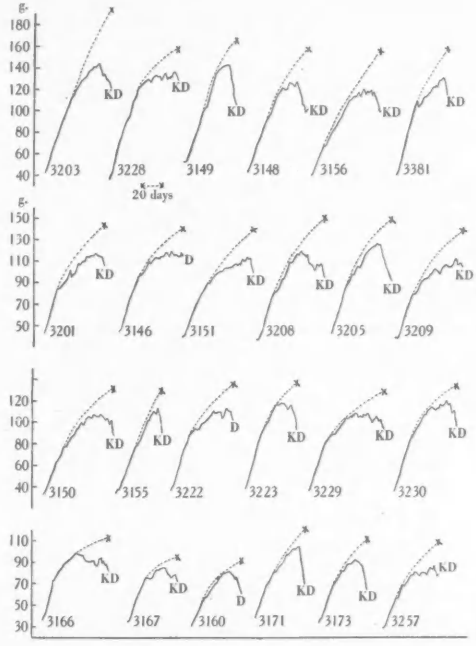



Fig. 4*a*. Weight curves and bone growth curves for rats receiving no vitamin A, showing the continuation of skeletal growth after weight increase has ceased.

Figs. 4 and 5 show the contrast between weight and bone growth for representative experimental animals, the corresponding numerical data being given in Tables V and VI. Fig. 4 *a* gives the graphs for 24 rats which were allowed to die or become moribund on the vitamin A-deficient diet. Both sexes are included, and the periods on the experimental diet vary from 42 to 70 days. The graphs have been arranged in groups in descending order of weight as estimated from skeletal development, the average weights for successive rows being 165·3, 145·2, 135·4 and 109·0 g. It will be seen that when vitamin A is completely absent from the diet, the discrepancy between bone growth and weight increase

is universal in its occurrence, whether the animals are well or poorly developed, whether death occurs suddenly, very soon after the first drop in weight, *e.g.* rats 3149, 3155 and 3171, or whether there is a fairly prolonged flattening of the weight curve, *e.g.* rats 3228, 3156, 3146, 3229 and 3166. Even in animals killed before reaching moribund condition, after only 4–5 weeks on the diet, the disproportion between bone length and body weight is already apparent (Fig. 4 *b*).

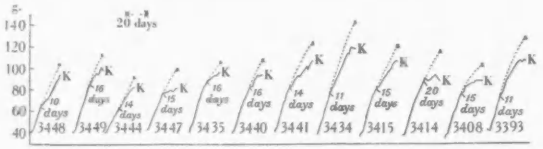



Fig. 4*b*. Curves showing early divergence of bone growth from weight. For the 12 rats above, which received no vitamin A, the average time of divergence is 14·4 days.

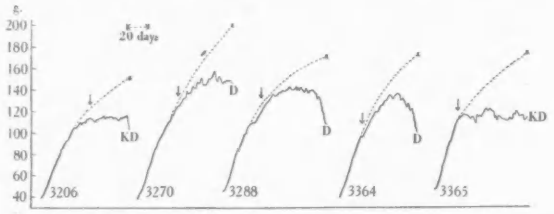



Fig. 5*a*. Divergence of bone growth from weight in rats receiving an inadequate dose of vitamin A. The arrow indicates the point at which vitamin A dosage was begun.

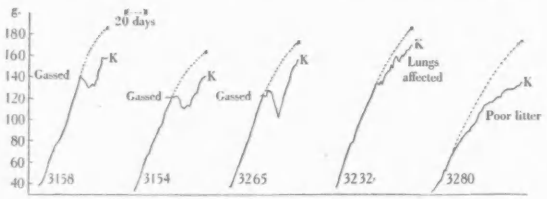



Fig. 5*b*. Divergence of bone growth from weight in rats receiving an adequate dose of vitamin A, but suffering from some pathological condition.

The course of the graphs seems to indicate that divergence between growth and weight may begin early, the average time of divergence in the 12 examples given being 14·4 days.

In Fig. 5 *a* are given the curves for 5 rats which, after varying periods (21–46 days) on the vitamin A-free diet, were given a small dose of vitamin A in the form of "fortified milk." In these rats, with their prolonged survival periods, the difference between bone growth and weight is marked. Thus in Rat 3365 we find that a practically constant weight has been maintained for 9 weeks, while the bones have continued to increase far beyond the length normally associated with this weight.

Fig. 5 *b* shows graphs for a series of 5 rats which received ample vitamin A, but which were in a pathological condition from varying causes, as indicated in

the diagram. The same disproportion between body weight and bone growth is apparent in these cases as was found in the animals suffering from vitamin A deficiency.

Disproportion between bone growth and body weight may apparently be produced by varying types of nutritional deficiencies, *e.g.* by restriction in the amount of food [Aron, 1911], mineral salt deficiency [Clarke and Smith, 1933], deficiency of vitamin A or vitamin B [Quinn *et al.*, 1929]. These last authors state that in these vitamin deficiencies there was less retardation of increase in skeletal measurements than in body weight, but they do not give their numerical data in a form which facilitates comparison with the values for their normal controls.

*Statistical analysis of the data for the vitamin A-deficient rats.* In order to have some estimate of the probable error of the weights calculated from the observed bone lengths, a statistical analysis has been carried out of the data for the 24 rats included in Table V, which were allowed to die or become moribund on the vitamin A-deficient diet. It was found (Table III) that these vitamin A-deficient rats were correlated to the same degree as the control rats, so far as weight of rat and length of bone are concerned. The curves of bone lengths on weights for the deficient rats are shown along with those of the control rats in Figs. 3 *a*, *b*, *c* and the corresponding numerical data in Table IV. In each case the predicted weights for length of bone of the deficient rats are less than the predicted weights for length of bone of the control series. Those values for vitamin A-deficient rats outside twice the standard error of the values for the control series may be regarded as quite significantly less than the controls. It

Table V. *Relation of actual weight to weight predicted statistically from bone lengths in vitamin A-deficient rats.*

Mean predicted weight = 138·74 g. Standard error of difference = 5·55
Mean actual weight = 114·04 g. Ratio = 4·45
Difference = 24·7 g.

| (1) V.A.D. rats (No.) | (2) Diet | (3) Period on diet days | (4) Actual lengths of bones | | | (5) Weights predicted from lengths of bones | | | (6) Av. of predicted weights | (7) Max. wt. of rat | (8) D = Difference in wt. (6)–(7) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Femur cm. | Tibia cm. | Humerus cm. | Femur g. | Tibia g. | Humerus g. | g. | g. | g. |
| 3160 ♀ | L+M | 47 | 2·46 | 2·86 | 1·94 | 96 | 94 | 94 | 94·67 | 81 | 13·67 |
| 3167 ♀ | L+M | 47 | 2·45 | 2·90 | 1·95 | 95 | 99 | 95·7 | 96·57 | 84 | 12·57 |
| 3173 ♀ | L | 45 | 2·60 | 3·02 | 2·04 | 113 | 115 | 115 | 114·33 | 91 | 23·33 |
| 3257 ♀ | L+M | 52 | 2·59 | 3·00 | 2·00 | 111·8 | 112 | 106·3 | 110·03 | 96 | 14·03 |
| 3166 ♀ | L | 62 | 2·61 | 3·05 | 2·03 | 114·2 | 119·3 | 113 | 115·50 | 98 | 17·50 |
| 3171 ♂ | L | 46 | 2·65 | 3·11 | 2·07 | 119·7 | 127·7 | 121 | 122·80 | 104 | 18·80 |
| 3150 ♀ | L | 65 | 2·77 | 3·15 | 2·12 | 137·2 | 133·8 | 132 | 134·33 | 107 | 27·33 |
| 3229 ♀ | L | 70 | 2·74 | 3·18 | 2·09 | 132 | 138·2 | 126 | 132·07 | 108 | 24·07 |
| 3209 ♂ | L+M | 63 | 2·80 | 3·24 | 2·12 | 142 | 147·2 | 132 | 140·40 | 111 | 29·40 |
| 3155 ♂ | L+M | 42 | 2·75 | 3·16 | 2·10 | 135·5 | 135·2 | 128 | 132·90 | 113 | 19·90 |
| 3222 ♂ | L | 58 | 2·78 | 3·23 | 2·11 | 139 | 146 | 130 | 138·33 | 113 | 25·33 |
| 3151 ♀ | L | 66 | 2·82 | 3·22 | 2·14 | 145 | 144·2 | 136·5 | 141·90 | 114 | 27·90 |
| 3201 ♀ | L+M | 56 | 2·81 | 3·24 | 2·18 | 143·3 | 147·3 | 146 | 145·53 | 117 | 28·53 |
| 3223 ♂ | L | 48 | 2·81 | 3·22 | 2·10 | 143·3 | 144·2 | 128 | 138·50 | 117 | 21·50 |
| 3146 ♀ | L+M | 60 | 2·78 | 3·25 | 2·15 | 139 | 149 | 139 | 142·33 | 119 | 23·33 |
| 3208 ♂ | L+M | 63 | 2·88 | 3·27 | 2·18 | 155 | 152 | 146 | 151·00 | 119 | 32·00 |
| 3156 ♂ | L | 64 | 2·89 | 3·33 | 2·21 | 156·5 | 162 | 152 | 156·83 | 120 | 36·83 |
| 3230 ♀ | L | 58 | 2·76 | 3·21 | 2·11 | 136 | 143 | 130 | 136·33 | 120 | 16·33 |
| 3205 ♂ | L+M | 56 | 2·86 | 3·28 | 2·18 | 151 | 153·7 | 146 | 150·23 | 126 | 24·23 |
| 3148 ♂ | L+M | 56 | 2·92 | 3·31 | 2·22 | 161·5 | 158·5 | 155 | 158·33 | 127 | 31·33 |
| 3381 ♂ | L | 47 | 2·94 | 3·30 | 2·21 | 165 | 157 | 152 | 158·00 | 130 | 28·00 |
| 3228 ♂ | L+M | 64 | 2·92 | 3·38 | 2·18 | 161·5 | 170 | 146 | 159·17 | 136 | 23·17 |
| 3149 ♂ | L | 49 | 2·98 | 3·34 | 2·26 | 172·2 | 163·8 | 165 | 167·00 | 142 | 25·00 |
| 3203 ♂ | L | 61 | 3·12 | 3·50 | 2·36 | 199 | 190·8 | 188 | 192·60 | 144 | 48·60 |
| | | | | | | | | | Mean = 138·74 | Mean = 114·04 | |

Table VI. *Relation of actual weight to weight predicted statistically from bone lengths in rats suffering from pathological condition due to vitamin A deficiency or other cause.*

| | (1) No. of rat | (2) Diet | (3) Period on diet days | (4) Actual lengths of bones: Femur cm. | Tibia cm. | Humerus cm. | (5) Weights predicted from lengths of bones: Femur g. | Tibia g. | Humerus g. | (6) Av. of predicted weights g. | (7) Max. wt. of rat g. | (8) Difference in wt. (6)–(7) g. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (*a*) | 3448 ♂ | L | 28 | 2·55 | 2·98 | 1·99 | 106·7 | 109·8 | 104·1 | 106·9 | 94 | 12·9 |
| | 3449 ♂ | L+M | 28 | 2·61 | 3·02 | 2·06 | 114·4 | 115·2 | 119·1 | 116·2 | 101 | 15·2 |
| | 3444 ♀ | L | 28 | 2·45 | 2·87 | 1·94 | 95·0 | 95·5 | 93·6 | 94·7 | 82 | 12·7 |
| | 3447 ♀ | L+M | 28 | 2·49 | 2·95 | 1·97 | 99·5 | 105·8 | 99·8 | 101·7 | 81 | 20·7 |
| | 3435 ♀ | L | 28 | 2·59 | 2·97 | 2·00 | 111·7 | 108·4 | 106·2 | 108·8 | 97 | 11·8 |
| | 3440 ♀ | L+M | 28 | 2·56 | 2·99 | 2·03 | 107·9 | 111·1 | 112·6 | 110·5 | 94 | 16·5 |
| | 3441 ♀ | L | 35 | 2·69 | 3·10 | 2·10 | 125·3 | 126·5 | 127·9 | 126·6 | 107 | 19·6 |
| | 3434 ♂ | L | 35 | 2·80 | 3·22 | 2·18 | 141·8 | 144·4 | 145·9 | 144·0 | 119 | 25·0 |
| | 3415 ♂ | L | 35 | 2·67 | 3·10 | 2·07 | 122·5 | 126·5 | 121·3 | 123·4 | 107 | 16·4 |
| | 3414 ♂ | L+M | 35 | 2·63 | 3·06 | 2·06 | 117·0 | 120·8 | 119·1 | 119·0 | 94 | 25·0 |
| | 3408 ♀ | L | 35 | 2·54 | 2·97 | 2·00 | 105·4 | 108·4 | 106·2 | 106·7 | 88 | 18·7 |
| | 3393 ♀ | L | 35 | 2·71 | 3·13 | 2·13 | 128·2 | 130·8 | 134·6 | 131·2 | 108 | 23·2 |
| (*b*) | 3206 ♀ | F.M. | 82 | 2·87 | 3·31 | 2·18 | 153·2 | 158·7 | 145·9 | 152·6 | 116 | 36·6 |
| | 3270 ♂ | F.M. | 89 | 3·15 | 3·51 | 2·38 | 205·4 | 192·8 | 193·3 | 197·2 | 157 | 40·2 |
| | 3288 ♀ | F.M. | 97 | 3·00 | 3·40 | 2·28 | 176·1 | 173·6 | 169·2 | 173·0 | 143 | 30·0 |
| | 3364 ♀ | F.M. | 73 | 3·03 | 3·37 | 2·29 | 181·7 | 168·6 | 171·5 | 173·9 | 136 | 37·9 |
| | 3365 ♂ | F.M. | 86 | 2·95 | 3·33 | Lost | 167·0 | 161·9 | — | 164·5 | 122 | 42·5 |
| (*c*) | 3158 ♂ | L+A | 63 | 3·05 | 3·45 | 2·35 | 185·5 | 182·2 | 186·0 | 184·6 | 158 | 26·6 |
| | 3154 ♀ | L+A | 66 | 2·93 | 3·38 | 2·24 | 163·5 | 170·2 | 159·7 | 164·5 | 140 | 24·5 |
| | 3265 ♀ | L+A | 63 | 2·96 | 3·42 | 2·30 | 168·8 | 177·0 | 173·9 | 173·2 | 156 | 17·2 |
| | 3232 ♀ | L+A | 70 | 3·07 | 3·43 | 2·35 | 189·4 | 178·7 | 186·0 | 184·7 | 169 | 15·7 |
| | 3280 ♀ | L+A | 84 | 2·98 | 3·39 | 2·32 | 172·4 | 171·9 | 178·7 | 174·3 | 135 | 39·3 |

will be noted that the result for the humerus is less significant than those for the femur and tibia. It may be that this fact is associated with the greater activity of the hind-limbs as compared with the fore-limbs in the rat.

In Table V the "standard" weights corresponding to the observed bone lengths are compared with the maximum weights actually attained by the vitamin A-deficient rats. In each case the actual weight was found to be distinctly less than the standard weight. The table shows the predicted weight, the actual maximum weight attained and the difference in weight for each vitamin A-deficient rat. The standard error of the difference between the two means was found to be 5·55. The difference between the two means was 138·74 − 114·04 = 24·7 g., which is 4·45 times the standard error and 6·6 times the probable error. These figures indicate clearly that the deficient rats are significantly less in weight than the controls for the actual lengths of bone of the vitamin A-deficient series. In other words, length of bone in the vitamin A-deficient series is significantly greater than in controls of the same weight, showing that the bones have continued to increase in length after increase in weight has ceased.

With regard to the 12 rats in Table VI, which were killed after 4–5 weeks on the deficient diet, the differences between predicted weights and actual weights are naturally not very large in most cases. Although these differences may possibly not be statistically significant, nevertheless when taken in conjunction with the data for the older rats, they may be regarded as showing that the divergence between bone growth and body weight tends to show at an early stage.

*Food consumption and retardation of growth in vitamin A deficiency.* It is clear both from the live measurements and from the measurements of bone length that growth in the sense of increase in length has not ceased in animals suffering from vitamin A deficiency, and although in most cases the rate of growth has been considerably retarded in the deficient rats, this is no justification

for assigning to vitamin A a specific influence on growth. Even with a diet fully adequate in quality, the rate of growth of an animal is largely dependent on the amount of food consumed, and we have found that the food consumption of rats on the vitamin A-free diet is much below that of the positive controls receiving vitamin A. This is shown graphically in Fig. 6, which gives weight and food con-

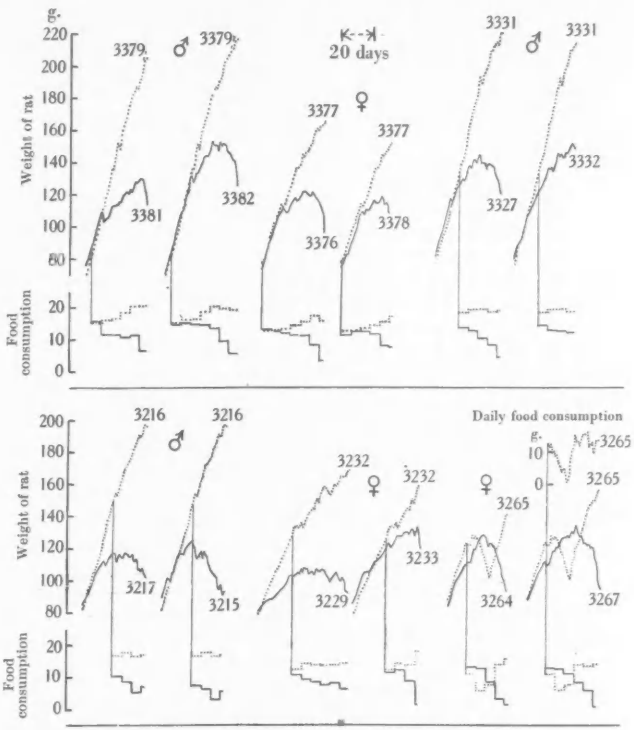



Fig. 6. Food consumption and weight graphs for pairs of litter-mates of the same sex, one rat on vitamin A-free diet (——), and the other on the same diet supplemented by vitamin A (· · · ·). The amount consumed has been averaged for periods of 6 days, except in the final period, which varies from 1 to 5 days. The food was given as a stiff paste formed into balls weighing 24 g. Similar balls left beside the cages overnight and weighed at the same time as the residues were found to weigh 22·0–22·3 g., with an average of 22·2 g. for 11 determinations. The weight of the residues was therefore subtracted from 22·2 g., and the food consumption was thus found approximately in terms of this dried paste, of which the actual moisture content was found by analysis to be 33 %.

sumption diagrams for 12 pairs of rats, each pair consisting of a positive control and a litter-mate on deficient diet. The food consumption records do not cover the whole period from weaning but were begun at varying stages of the deficiency, as shown in the diagram, and continued until death.

A study of the graphs shows that food consumption is practically identical for the control and deficient rats during the period when the graphs of weight are in close agreement (*e.g.* during the first 6–12 days of the food records in the first

4 pairs of rats), and that the beginning of divergence in the weight curves coincides with a corresponding divergence in the amount of food intake. Even in rats receiving vitamin A, the food intake is influenced very considerably by their physical condition. Thus for rat 3232 ♀, in which the lungs were affected, the food intake remained practically constant for 28 days at approximately 14 g., while the intake for a more normal female control, *viz.* 3377, rose steadily in the same period from 13 to nearly 18 g. Similarly in rat 3265, which suffered severely from gassing and showed a very sharp drop in weight, the food intakes in the first 3 periods were less than those for the two litter-mates receiving no vitamin A (pairs 11 and 12), and the subsequent rise in the weight curve, and also in the growth curves as previously noted, was accompanied by a return to normal food intake.

The daily food consumption has been recorded graphically for rat 3265 to illustrate the very close correspondence found between weight fluctuations and food intake. Our findings in this respect agree with those of Irwin *et al.* [1930], who showed that in vitamin work variability in weight gain was largely due to variations in total food intake. Sampson *et al.* [1932] found that in vitamin A deficiency the decrease in food intake could offer only a partial explanation of the change in rate of growth. It is to be noted, however, that they calculate food consumption per 200 g. body weight and estimate growth by increase in weight. Our work has shown that in pathological cases, such as occur in vitamin A deficiency, weight cannot be regarded as an accurate index of the growth of the animal. Hence any comparison of food intakes and metabolism on the basis of body weight may lead to fallacious conclusions.

The data we have recorded indicate clearly that the faster growth attained by the rats receiving vitamin A depends to a large extent on their greater food consumption, and that the appetite of all the rats is determined largely by their state of health. If an animal falls into ill-health, even when it is receiving vitamin A, appetite fails and the rate of growth slows down. Similarly in animals deprived of vitamin A the pathological conditions caused by this deprivation are responsible for the diminished appetite, the consequent fall in weight and the slower rate of growth.

The existence of pathological conditions in vitamin A deficiency has been noted by most workers, but they occur much earlier than has been generally supposed. Recently Thatcher and Sure [1932] found that "71 % of the animals deficient in vitamin A showed metaplastic changes before decline of body weight or complete cessation of growth took place." About one-third of their animals were killed after 33–40 days on the diet; the remainder at a later date. We have found that the pathological condition begins even earlier than this. In animals killed after being only 21 days on the diet, such conditions as ulceration of the stomach or caecum, and inflammation of the caecum or small intestine, especially the duodenum, could be detected macroscopically in over 70 % of the cases.

It would appear that the animal's capacity for storing reserves of vitamin A is much less than is generally supposed, and that the preliminary or "depletion" period, during which it is assumed to be using up these reserves is in reality the time taken for the outward manifestation of symptoms of disease which may be already far advanced. The relationship between pathological conditions and the weight responses obtained in the curative method of vitamin A assay is discussed in detail in a subsequent paper [Richards and Simpson, 1934]. There is little doubt that the diversity of these conditions in the experimental animals after the so-called "depletion" period accounts for most of the discrepancies encountered in such assays.

## Conclusions.

A survey of the above experimental data indicates that there is no ground for ascribing any special growth-promoting activity to vitamin A. The characteristic loss of weight occurring in its absence does not indicate cessation of growth as has been generally assumed but is a sign of the onset of disease. Vitamin A is merely one of the many dietary components, known and unknown, that are essential for optimum development and growth in the organism. In this sense only is vitamin A a "growth factor," and its claim to the title is no greater than that of any other indispensable dietary constituent. Its power of preventing disease remains beyond question, although the term "anti-infective" vitamin proposed by Green and Mellanby [1928] has not met with general approval as an accurate definition of its function. It seems wiser for the present, in the words of Mendel [1932] "to stress the indefinite function of the vitamin in preserving health and vigour, rather than to herald any specific action against definite microbiotic enemies."

## Summary.

1. Measurements of tail length and total body length made at intervals on rats suffering from avitaminosis-A have shown that increase in length continues when increase in weight has ceased.

2. *Post mortem* measurement of the limb bones has shown that the relationship between body weight and bone length established for the positive controls is not maintained in the vitamin A-deficient animals. The bones have continued to increase in length while the animal has been losing weight. The weight curve is thus not a true index of skeletal growth.

3. The results recorded here indicate that the most important rôle of vitamin A in nutrition lies in its power of preventing the onset of pathological conditions, which are the cause of the loss of weight that occurs in vitamin A deficiency.

The authors are indebted to Dr J. F. Tocher of Aberdeen University for carrying out all the statistical work involved in this investigation.

They wish also to express their thanks to Miss B. W. Simpson for valuable assistance with the experimental work.

## REFERENCES.

Aron (1911). *Philippine J. Sci. B.* (*Med. Sci.*), **6**, 1.
Busson (1933). Medical Thesis, Paris. (Abstracted in *Nutrition Abstr. Rev.* 1933, **3**, 391.)
Clarke and Smith (1933). *Amer. J. Physiol.* **105**, *Proc.* 20.
Green and Mellanby (1928). *Brit. Med. J.* ii, 691.
Hopkins (1912). *J. Physiol.* **44**, 425.
Irwin, Brandt and Nelson (1930). *J. Biol. Chem.* **88**, 449.
Kuhn and Brockman (1933). *Ber. deutsch. chem. Ges.* **66**, 407.
Medical Research Council (1932). Vitamins: a survey of present knowledge. *Med. Res. Coun. Spec. Rep. Series*, No. 167.
Mendel (1932). *J. Amer. Med. Assoc.* **98**, 1981.
Orr and Richards (1934). *Nature*, **133**, 255.
Osborne and Mendel (1913). *J. Biol. Chem.* **15**, 311.
Quinn, King and Dimit (1929). *J. Nutrition*, **2**, 7.
Richards and Simpson (1934). *Biochem. J.* **28**, 1274.
Sampson, Dennison and Korenchevsky (1932). *Biochem. J.* **26**, 1315.
Steenbock and Nelson (1923). *J. Biol. Chem.* **56**, 365.
—— —— and Black (1924–25). *J. Biol. Chem.* **62**, 275.
Thatcher and Sure (1932). *Arch. Path.* **13**, 756.

# CLXXIII. THE CURATIVE METHOD OF VITAMIN A ASSAY.

By MARION BROCK RICHARDS and BEATRICE WEIR SIMPSON.

*From the Rowett Research Institute, Aberdeen.*

(*Received July 2nd, 1934.*)

The curative method of vitamin A assay, which is still the method most widely used for the biological estimation of vitamin A, is thus summarised in the Medical Research Council's Report on "Vitamins" [1932]: "In a curative test, the animals are allowed to exhaust their reserves of vitamin A, as indicated by cessation of growth, after which doses of the test substance are administered for about 5 weeks.... Comparisons are drawn between doses which give equal average increments in weight." The method is apparently simple, but reference to the literature makes it evident that in practice the results obtained are far from satisfactory. Serious discrepancies occur not only among the results obtained by workers in different laboratories, but even among those obtained in one laboratory by the same workers using carefully standardised procedure. Thus Drummond and Morton [1929], in analysing the results obtained for the vitamin A content of a series of oils, give the case of two litter-mates of which one lost 24 g. in 4 weeks while the other, on the same dose of cod-liver oil, gained 34 g. in 5 weeks. Such extreme variations are by no means infrequent in their occurrence and could probably be paralleled by most workers from their own data.

The history of vitamin A assay is largely a record of attempts made to eliminate such discrepancies. In order to secure uniformity in the experimental animals stress has been laid on the necessity of using litter-mates for comparison, and of reducing the reserve stores of vitamin A in the young by careful regulation of the mother's diet. Certain workers find it preferable to take the occurrence of xerophthalmia rather than "cessation of growth" as an indication of depletion of the animal's reserve stores of vitamin A. Coward *et al.* [1930] have emphasised the importance of the type of caseinogen used in the basal diet. They found that by substitution of extracted "light white casein" for the "vitamin-free casein" previously used, it was possible to obtain greater uniformity in the results and to get growth-response graded to the dose of vitamin A.

The failure of all such attempts to effect the necessary improvement in results, the growing dissatisfaction with the method among experienced workers and their striking lack of agreement in regard to details of technique are indicated clearly by papers published in 1933 from three different English laboratories. Thus Culhane [1933], in reporting work with various basal diets, states that rats on her vitamin A-free diet invariably develop xerophthalmia, whereas Bacharach [1933] finds the onset of xerophthalmia so uncertain that it is useless as a criterion of avitaminosis-A. Further, Bacharach's results, "in their bearing on the source of protein in vitamin A-free diets, do not confirm those reported by Coward," and Culhane from her experiments comes to the conclusion that Coward's "light white casein" contains some vitamin A. Of even greater significance is a communication by Coward *et al.* [1933], in which they report the

great variations they have themselves found in the apparent vitamin A value of a sample of cod-liver oil, when tested at different times. Their numerical values for one sample, expressed as abscissae of the curves of response relating weight increase to dose of vitamin A, range from 0·73 to 3·48. After investigating possible causes of the variation in response, such as seasonal influence, changes in basal diet and differences in the weight of the rats when depleted of vitamin A, the authors conclude that the variation must be due to "some influence not yet determined."

From a review of the literature it would appear that some fundamental source of error in the technique of the assay has been overlooked. The outline of the method given shows that the technique is based entirely on the "growth-promoting property" of vitamin A, "cessation of growth" being regarded as the criterion of depletion of the reserve stores, and "resumption of growth" after addition of a supplement as an indication of the presence of vitamin A in that supplement [Medical Research Council, 1932]. The significance of the pathological conditions that arise from vitamin A deficiency does not seem to have been taken sufficiently into account. Obvious signs of disease such as xerophthalmia and lung trouble have received attention, while other more dangerous but less apparent symptoms have been almost ignored. The occurrence of diverse pathological conditions has been reported by numerous workers, notably by Green and Mellanby [1928], but apparently those who have been interested in the relationship of vitamin A to disease have not as a rule been concerned with the quantitative estimation of the vitamin, while the assay workers have not recognised fully the extent to which their results can be affected by the varied nature of the symptoms arising in the absence of vitamin A.

In the course of a number of assays, by the curative method, of the vitamin A content of various samples of fish oils and dried milk, we have accumulated much evidence regarding the unsatisfactory nature of the curative test. In addition to many discrepancies of the types frequently reported in such tests, difficulties of another nature have been encountered. Much of the evidence could not be reconciled with current ideas regarding the growth-promoting activity of vitamin A and the cessation of growth that is said to occur after the animal's reserve stores of the vitamin have been depleted. It was clear that in many cases, particularly in the dried milk experiments, the weight curve did not give a reliable indication of the state of depletion of the animal, and that increase in weight after administration of a supplement could by no means invariably be ascribed to the vitamin A content of that supplement. Consequent on these observations experiments were carried out in this Institute which proved that there is in reality no cessation of growth in vitamin A deficiency [Orr and Richards, 1934]. The animal continues to increase in size, as determined by skeletal development, even when it ceases to increase in weight, and the fall in weight which eventually occurs is simply the result of the pathological conditions due to the vitamin deficiency.

The present paper is a summary of experimental data bearing on various points of technique in the curative method. The results illustrate the errors which may arise from taking the weight curve either as an index of vitamin A depletion in the preliminary period, or as a measure of vitamin A potency in the test period. There is little room for doubt that the cause of many of the reported discrepancies lies in the different pathological conditions of the experimental animals at the end of the preliminary period.

## Experimental.

### *Weight curve during the preliminary period.*

The weight curve during the preliminary period has been taken by most workers to indicate the state of depletion of the animal's reserve stores of vitamin A. As these stores become exhausted the weight curve tends to flatten, and dosing with vitamin A is begun when it is clear that weight increase has ceased. Considerable importance has been attached in curative tests to the actual weights attained by the rats during the preliminary period, and it is customary to reject from the test animals which grow beyond a certain limit. The Medical Research Council's Report states that "suitable" animals cease to grow at about 100 g. weight. Coward *et al.* [1932, 1] consider that the rats should cease to grow at 80–100 g. and they regard 110 and 120 g. as the permissible maxima for females and males respectively.

We have found that the course of the weight curve during the pre-period is influenced very significantly both by the composition of the basal diet, quite apart from its lack of vitamin A, and by the type of experimental animal used. On a given basal diet, the weight level at which the curve tends to flatten or fall suddenly will be higher for a vigorous animal than for one of a more weakly type, while the use of a more complete diet will tend to raise the weight level of all the rats.

*Composition of the basal diet.* In selecting the basal diet the tendency of assayers seems to have been to direct attention chiefly to the necessity of using a diet completely devoid of vitamin A, and to ignore the equally important requirement that "all growth-promoting factors other than the one under test should be abundantly supplied" [Medical Research Council, 1932]. Unless this requirement is satisfied, retardation in the rate of growth will not necessarily be due to lack of vitamin A, and increase in weight may be caused by the administration of other missing factors. While the theoretical ideal may as yet be impossible of attainment, in view of our present imperfect knowledge of nutrition, the diet should at least be so chosen as to include all known essential factors other than vitamin A. Throughout this series of experiments, except in a preliminary oil assay, we have used with slight modifications the vitamin A-free diet recommended by Sampson *et al.* [1932]. This diet (Diet L), which includes fat and a high proportion of dried yeast, seems to approximate more closely to the theoretical ideal than some of the basal diets in use.

As an illustration of the extent to which the weights attained by the rats in the pre-period can be influenced by the composition of the basal diet, the following data may be given. In a preliminary oil assay, in which the basal diet used (Diet I) consisted of caseinogen 20, rice starch 70, salts 5 and yeast 5, with radiostol as source of vitamin D, 14·4 % of a lot of 104 rats exceeded the limits of weight set by Coward and should therefore have been rejected as "unsuitable" for experiment. In a later assay, in which basal Diet L was used, 57 % of a batch of 51 rats of similar breeding should have been rejected by the same standards. Diet L consisted of caseinogen 18, wheat starch 50, cotton-seed oil 15, salts (McCollum 185) 5, dried yeast 18·3 and lemon juice 5, with a small amount of potassium iodide solution and sufficient water to make into a stiff paste. Vitamin D was supplied as radiostol (0·02 ml. per rat twice weekly). That the superior "growth-promoting" properties of Diet L are not due to any accidental inclusion of a source of vitamin A may be seen from the weight curves of 12 rats which served as negative controls in this series of experiments (Fig. 1). These animals received throughout, after weaning at 21–25 days, the basal diet together

with a supplement of 0·075 g. of dried separated milk (Diet L+M). The nature of the curves and the lengths of the survival periods (28–53 days for the males and 47–70 days for the females) furnish strong evidence that the amount of vitamin A, if any, in the basal diet and in this amount of dried milk is entirely

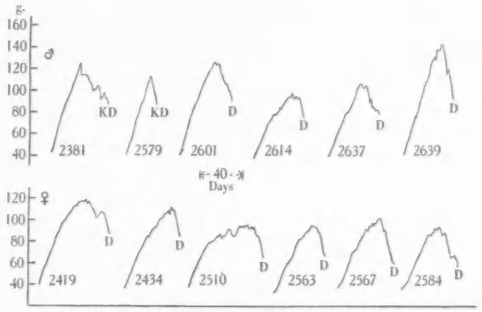



Fig. 1. Negative controls. Basal diet supplemented with 0·075 g. "unfortified milk," *i.e.* dried separated milk, which gave a negative result when tested colorimetrically for vitamin A. KD = killed in moribund condition; D = died.

negligible. In addition to the effect on the weight curves, the substitution of Diet L for Diet I caused a marked improvement in the general condition of the animals. In rats fed on Diet L there were none of the sores on feet, tail, shoulders and neck which were of frequent occurrence in the earlier experiment. There is little doubt that Diet I was too low in vitamin B, and that the improved results on Diet L must be attributed largely to its exceptionally high proportion of yeast, amounting to about 20 % of the dry constituents. The inclusion of fat is probably also a factor of importance, since Culhane [1933] has shown that body sores occur more readily on a fat-free diet.

*Type of experimental animal.* No adequate reason seems to have been given for rejecting from the test animals which grow beyond the prescribed limits of weight during the preliminary period. Even among litter-mates it frequently happens that from the beginning certain individuals show greater vitality than others. These rats have a tendency to maintain appetite and weight-increase beyond the level at which the less vigorous litter-mate with its more capricious appetite tends to fall off. The practical result of the weight limitation imposed would thus appear to be to eliminate from experiment the more vigorous animals. Such animals may possibly be less likely to give the desired response to vitamin A dosage, but this seems scarcely sufficient cause for rejecting them, and for confining the tests to animals of a less vigorous type. The minimum effective dose of vitamin A determined for a weakly rat of poor growth rate will not necessarily be effective for a more vigorous animal, and a biological method cannot be regarded as satisfactory if its results are applicable only to a limited class of animals of a subnormal standard.

*Weight curve during the test period.*

In the curative method increase in weight during the test period is regarded as an indication of the presence of vitamin A in the substance tested. Even small temporary increases in weight occurring when a vitamin A-free diet has been replaced by another basal diet have been taken as proof that the second diet

contains some vitamin A. Little attention has been paid to the fact that with animals kept for some time on one ration, practically any change in the diet will stimulate appetite, temporarily at least, and if the changed diet be particularly palatable, the effect on the weight curve may be of considerable duration, even though the added substance is devoid of vitamin A.

Results of this type occurred in an assay of "fortified milk," *i.e.* dried separated milk to which had been added before evaporation a vitamin A concentrate. A blue value test carried out on the ether extract of the milk sample had indicated that apparently only a small amount of the vitamin A potency of the added concentrate had been retained during evaporation. When the biological test was carried out by the usual curative procedure (Curative method I), *i.e.* giving the milk as a supplement after a preliminary period on the basal diet, the weight curves in a number of cases seemed to show the presence of a considerable amount of vitamin A in the "fortified milk," and no fewer than 20 of the 30 rats on the three doses fed (0·025, 0·050 and 0·075 g. milk) survived the test period of 5 weeks. The condition of the rats, however, made it evident that the amount of vitamin A present must have been small. Xerophthalmia when present was not cured, and in spite of considerable increases in weight, respiratory trouble developed later in several cases. In view of the obvious ill-health of the animals, it was felt that the prolonged survival periods and the increases in weight were not truly indicative of the amount of vitamin A in the "fortified milk," but were to be ascribed partly to the food value of the milk and partly (possibly chiefly) to the stimulating effect of the milk supplement on appetite. For administration the milk powder was mixed with water, and the requisite doses, which were given from a graduated pipette, were invariably taken with great avidity by the rats, even when they were practically moribund.

In order to eliminate the effect of the stimulus to appetite caused by the sudden addition of this extremely palatable supplement, the assay was repeated, using a slightly modified technique (Curative method II). Each rat received during the preliminary period a dose of "unfortified milk" (*i.e.* dried separated milk) equal in amount to the dose of "fortified milk" it was to receive during the test period. Thus any stimulus to appetite due to the milk supplement was present throughout the experiment, and any weight increase found after substituting "fortified milk" for "unfortified" could therefore be more legitimately ascribed to the vitamin A potency of the former. The striking difference in results brought about by this slight modification in procedure is shown in Fig. 2. In each row the first 3 curves are weight curves obtained by the ordinary curative method, while the remainder were obtained by the Curative method II. In place of the prolonged survival periods and the weight increases obtained by Curative method I, there is a marked preponderance of curves of the sharply peaked type. Only two out of 36 rats survived the test period of 5 weeks, and of these survivors one (graph not included in Fig. 2) was then unable to respond to a large daily dose of vitamin A concentrate. It survived only 10 days longer, and *post mortem* examination revealed tongue abscess and advanced pyelitis. In the other survivor (Rat 2441), which was killed after 95 days' dosing with vitamin A, the caecum was found to be highly distorted and perforated by an abscess.

The marked contrast between the results obtained in these two assays of "fortified milk" served to bring into prominence the mistaken assumptions on which the curative technique is based. In the first assay the ill-health of the animals made it clear that the weight-increases in the test period could not be regarded as indicative of vitamin A potency, and in the second assay, in a large

number of cases there was no sign of the occurrence of the growth plateau which is supposed to indicate the depletion of the animal's reserve stores of vitamin A. *Post mortem* examination of the rats showed the presence of disease of widely varying types, and it was obvious in many cases, especially where the weight

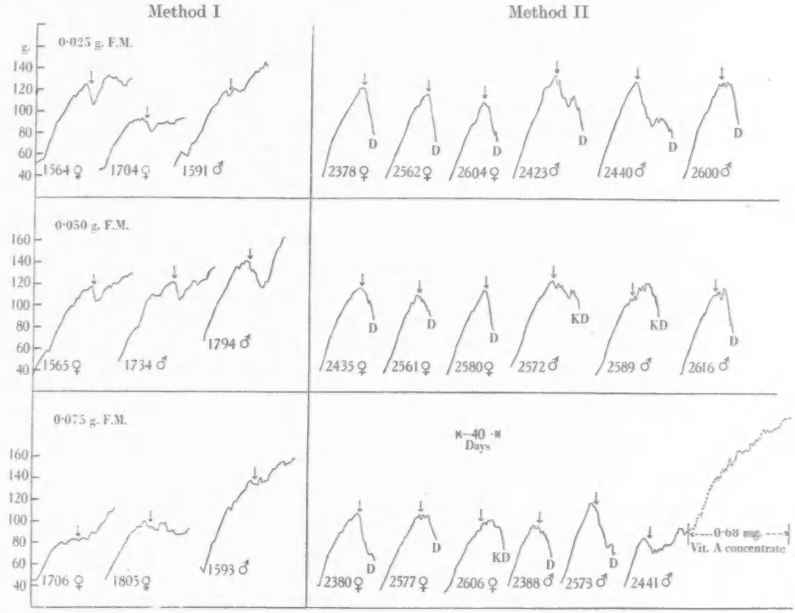



Fig. 2. Assay of "fortified milk" (F.M.) by the curative method.

*Method I*: Basal ration during preliminary period; supplement of "fortified milk" in test period.

*Method II*: Basal ration + "unfortified milk" during preliminary period; basal ration + "fortified milk" in test period.

curve was very sharply peaked, that disease must have been already far advanced while the weight curve was still rising steadily. It seemed evident that deficiency of vitamin A does not affect the process of growth *per se*, but that the inevitable sequel to such deficiency is the onset of disease, the effect on the weight curve being merely a secondary and incidental manifestation. The occurrence of a growth plateau seems to be more or less fortuitous, and in many cases no effect on the weight curve may be apparent until after ineradicable disease has been established.

Examples of the sharply peaked type of curve occur not infrequently even in ordinary curative tests, and it will usually be found that in such cases death ensues without any apparent response to the dose of vitamin. It is significant that any addition to the basal diet which renders it more complete seems to increase the number of such curves. Thus, for example, when Drummond *et al.* [1925] modified their technique of vitamin A assay by introducing a source of vitamin D into the diet, they found a tendency for curves to be sharply peaked

instead of falling off gradually. In our experiments a similar effect was produced by the change from Basal diet I to Diet L. The exceptionally high proportion of such curves recorded in the second assay of "fortified milk" might possibly therefore be an indication that the milk supplement has acted as a stimulus to appetite by supplying some unknown deficiency in the basal ration. On the other hand we have had many instances of sharply peaked curves, especially in males of the more vigorous type, when no milk supplement was given with the basal ration, while less vigorous rats, even when receiving a milk supplement, have shown a tendency to give flatter curves. Apparently the more vigorous the rat and the more complete the diet, the greater is the probability that a sudden decline in weight will set in without the usual premonitory flattening.

*Estimation of the end of the depletion period.*

One of the main difficulties in carrying out a curative assay is to determine when to begin administration of the vitamin. "Cessation of growth" is the criterion given in the M.R.C. Report, while in Sherman's technique, which seems to be that adopted by most American workers, the instructions are to "begin the test period a few days after growth has ceased on the basal diet, and about one day before the appearance of distinctly sore eyes....Have the animals thoroughly depleted of surplus vitamin A but not in a seriously pathological condition when the experimental period is begun" [Sherman and Burtis, 1928].

The occurrence of sharply peaked curves, such as those shown in Fig. 2, makes it clear how unsatisfactory are any instructions based on the course of the weight curves. In those cases the dose of vitamin A given was small, but even when a considerable or large dose is given, many instances of similar curves are found (Fig. 3 *a*). In such cases it is evident that no dose of vitamin A, however large, could have effected a cure. The impossibility of controlling the severity of the pathological conditions seems self-evident.

Certain workers have found it useful to take the ophthalmic reaction as an indication of depletion [*e.g.* Steenbock and Coward, 1927], but as has already been noted, there is a lack of unanimity regarding the invariability of its occurrence. If it does happen to occur, xerophthalmia is undoubtedly more reliable than the weight curve as an indication of avitaminosis-A. Certain data from one of our experiments are of interest in this connection. Pairs of litter-mates were placed on deficient diets, one rat receiving Diet L and the other Diet L+M. In spite of considerable differences in the weight curves during the preliminary period, the first signs of xerophthalmia when it did occur, appeared in litter-mates on approximately the same dates (Fig. 3 *b*). In the case of rats 3144 and 3145, the former, in which the eyes remained unaffected for a further period of 12 days, showed symptoms of lung trouble on the same day as xerophthalmia was first noted in its litter-mate. Reference to the graphs in Fig. 3 *b* shows that if these rats had been used in a curative test, with xerophthalmia as the index of depletion, dosing would have been begun in most cases several days sooner than would have been the case if the weight curve were taken as the index.

In estimating the end of the depletion period individual workers apparently use their own discretion in determining when to begin dosing, and in this one point there is room for a variability which may exert a powerful influence on the results obtained. It is obvious that the earlier dosing begins, the greater is the chance that the pathological conditions present will still be amenable to treatment, and the better will be the weight response. The weight curves of the two

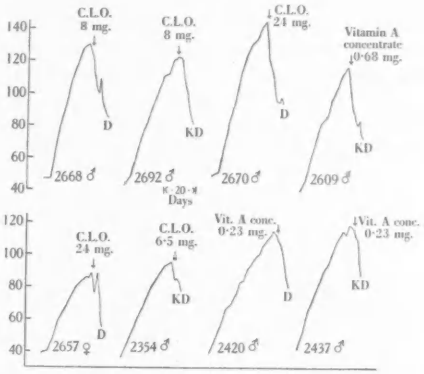



Fig. 3 *a*. Graphs illustrating the difficulty of estimating the end of the "depletion" period. Chief *post mortem* findings:

2668. Ballooning of intestines and caecum; pneumonia.
2692. Gastric haemorrhages; inanition.
2670. Stomach—keratosis and ulceration of non-glandular part; punctuate petechial areas all over glandular part.
2609. Caecum ballooned, 2 ulcers; peritonitis; stomach—many small ulcers.
2657. Ballooning of stomach and intestines; pneumonia.
2354. Pneumonia.
2420. Ballooning of stomach and intestines. (Noted before death.)
2437. Large tongue abscess; inanition.

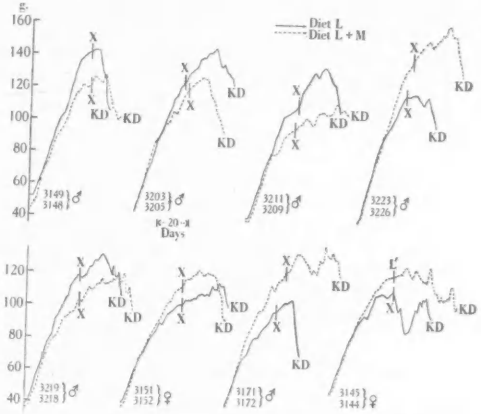



Fig. 3 *b*. Estimating the end of the "depletion" period. X = appearance of xerophthalmia; L' = symptoms of lung trouble.

series of male rats given in Fig. 4 illustrate the variations in response which accompany variations in the length of the preliminary period. In the first series each rat received the same dose of a vitamin A concentrate, after preliminary periods varying from 24 to 40 days. In the second series each rat received 2·2 mg.

of a good cod-liver oil after preliminary periods of 29–42 days. In both series it will be seen that where dosing was begun fairly early, *i.e.* after about $3\frac{1}{2}$–$4\frac{1}{2}$ weeks on the basal diet, the weight response was in general satisfactory, and on *post mortem* examination at the end of the test period in most cases no macro-

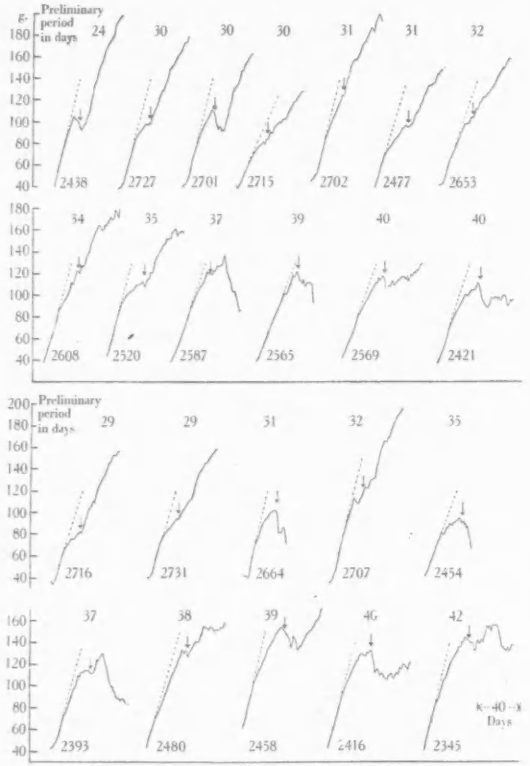



Fig. 4. Variations in response accompanying variations in the length of the preliminary period. The 13 rats of the first series each received 0·46 mg. of a vitamin A concentrate; the 10 rats of the second series received 2·2 mg. cod-liver oil. The dotted lines indicate the early stage at which the weight graph begins to diverge from the normal course.

scopic lesions were found. On the other hand, where dosing had been delayed until 34–42 days, the weight response was much poorer, and *post mortem* examination revealed the usual macroscopic evidence of disease, even after 5 weeks' dosing with vitamin A. Comparison of the *post mortem* findings given in Table I with the course of the weight curves in Fig. 4 shows how close is the connection between the weight response of the animal and its pathological condition. In the second series rat 2664, which died without any indication of response to the dose of vitamin, forms a good example of the risks inherent in the curative method. Even when dosing is begun as early as 31 days, the damage done may happen to be irreparable.

Table I. *Effect on pathological condition of varying the length of the depletion period in curative assays.* (*Cf.* Fig. 4.)

| No. of rat | Length of depletion period Days | Chief *post mortem* findings (macroscopic) (after 5 weeks' test period) |
|---|---|---|
| 2438 | 24 | No macroscopic lesions |
| 2727 | 30 | ,, |
| 2701 | 30 | ,, |
| 2715 | 30 | ,, |
| 2702 | 31 | ,, |
| 2477 | 31 | Inflammation of caecum |
| 2653 | 32 | No macroscopic lesions |
| 2608 | 34 | Stomach—2 healed ulcers. Caecum—ulcer |
| 2520 | 35 | Caecum—ulcer, and patch of chronic inflammation |
| 2587 | 37 | Caecum very badly ulcerated. End of ileum ulcerated all over. Inflammation of small intestine. Stomach—much keratosis, 4 ulcers |
| 2421 | 40 | Abscess on head. Large lung abscess |
| 2569 | 40 | Tongue abscess. Caecum—chronic ulceration. Inflammation of ileum |
| 2565 | 40 | Lungs badly infected. Intestines inflamed. Caecum ballooned |
| 2716 | 29 | Stomach—healed ulcer |
| 2731 | 29 | No macroscopic lesions |
| 2664 | 31 | Died after 9 days' dosing. Lungs infected. Inflammation of small intestine, including duodenum. Caecum ballooned |
| 2707 | 32 | No macroscopic lesions |
| 2454 | 35 | Large tongue abscess. Bladder abscess. Died after 8 days' dosing |
| 2480 | 36 | Duodenum inflamed. Entire caecum inflamed and distorted |
| 2393 | 36 | Large tongue abscess. Lung abscess. Caecum—large perforated ulcer |
| 2458 | 39 | Caecum—two ulcers |
| 2416 | 40 | Peritonitis. Caecum—healed perforated ulcer |
| 2345 | 42 | Stomach—keratosis. Duodenum—ulcer |

From these data it is evident that variations of even a few days in the length of the depletion period may cause wide discrepancies in the response of the animal to vitamin A dosage, and it is probable that such variations are the cause of some at least of the differences in the results obtained by different workers. Thus Dutcher *et al.* [1927] reported that they could get weekly gains of only 8–9 g. on 3 ml. of cow's milk, whereas Macy *et al.* [1927, 1] had got gains of 12–13 g. They suggested that the environment and diet of the cows might account for the difference, but a study of their technique indicates that the length of their preliminary period exceeded that given by Macy and co-workers by about 7–9 days, a prolongation which would have a very significant effect on the animal's response. The latter authors, who were evidently well aware of the dangers involved by the use of an extended preliminary period, advocated that curative feeding should commence before definite xerophthalmia and maintenance or loss in weight were observed, to avoid dealing with grossly pathological animals.

*Diversity of pathological conditions after preliminary period.*

Apart from the discrepancies that may arise from variations in the length of the preliminary period, a further source of error obviously lies in the wide diversity of the pathological conditions that may result from the vitamin A deprivation. As an illustration of this diversity we give in Table II the chief *post mortem* findings for the rats in the second milk assay, whose weight curves were included in Fig. 2.

Table II. Post mortem *findings in rats from second milk assay, showing diversity in pathological condition after depletion.* (*Cf.* Fig. 2.)

| | |
|---|---|
| 2378 | Tongue abscess; ballooning of stomach and intestines; peritonitis |
| 2562 | Ballooning and inflammation of entire intestine |
| 2604 | Inflammation of duodenum; pyelitis; cystitis |
| 2423 | Submaxillary abscesses; stomach—ulceration and keratosis; cystitis |
| 2440 | Tongue and submaxillary abscesses; stomach—keratosis, haemorrhage; caecum—perforated ulcer |
| 2600 | Tongue abscess; lungs infected; pus in seminal vesicles |
| 2435 | Pyelitis; cystitis |
| 2561 | Pyelitis; duodenal haemorrhages |
| 2520 | Stomach—keratosis; caecum ballooned; small intestine inflamed |
| 2572 | Neck and tongue abscesses; stomach—ulceration: caecum—inflammation; pyelitis |
| 2589 | Stomach—keratosis; caecum—inflammation; cystitis |
| 2616 | Stomach—keratosis; duodenum and small intestine highly inflamed; pus in seminal vesicles |
| 2380 | Caecum ballooned; pyelitis |
| 2577 | Pyelitis; bladder—ballooned, abscess |
| 2606 | Pyelitis; stomach—keratosis and ulceration: caecum—ulcer and inflammation |
| 2388 | Stomach and intestines ballooned |
| 2573 | Stomach—keratosis and inflammation |
| 2441 | Stomach—keratosis and ulceration; caecum—perforated ulcer |

A study of these findings brings into prominence one of the most radical defects of the curative method. In a biological test it is essential that the experimental animals at the commencement of the experiment should be as nearly alike in all respects as possible, and in vitamin A work much stress has been laid on the importance of securing uniformity in the animals at weaning. In a curative test, however, the real beginning of the experiment is not the time when the rats are first placed on the deficient diet, but the end of the "depletion" period, when vitamin A dosage is begun. This is the point, therefore, at which the animals should be in similar condition, if similarity in response is to be expected. It is significant that all assay workers reject from their tests animals which die during the early stages of dosing. Drummond and Morton [1929] reject all rats which die in the first 3 weeks. Coward *et al.* [1930] reject all those which die in the first week, since the number of these bears no relation to the dose of vitamin given. "Considering the varying and unknown degree of severity of the pathological condition of the animals," it is stated, "this is not surprising." It is to be noted, however, that these varying pathological conditions affect not only the rats which die during the first week of the test but also the survivors, and on the growth, or more correctly the weight-increase, of these survivors the calculations of vitamin A potency are based. It is obvious that the response of such animals to a dose of vitamin A must depend largely on the nature and severity of the pathological conditions present, and that these dissimilarities in the experimental material when dosing begins cannot fail to produce variations in the response of individual rats to the same dose of vitamin A. The various discrepancies reported by numerous assayers admit of easy interpretation if the possible pathological condition of the animal is taken into account.

*Persistence of pathological conditions.*

We have selected from our data numerous examples of "anomalous responses" which seem similar in type to results reported in the literature. These are presented along with the main findings of the *post mortem* examinations which were carried out on the rats, either when they became moribund as the result of the deficiency, or at the end of the 5 weeks' test period.

(1) *Appreciable growth at first, followed by sudden decline.* Ahmad and Drummond [1930] reported cases of this kind. Again, Culhane [1933] has stated that occasionally rats on a vitamin A-free diet "died rather suddenly without any obvious cause." In Fig. 5 the first 3 curves represent rats in which

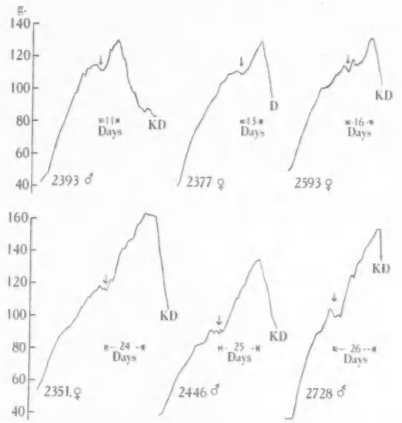



Fig. 5. Anomalous responses in vitamin A curative tests. Initial response followed by a decline. This type of result has been ascribed to incomplete depletion of the vitamin reserve during the preparatory period. The pathological conditions arising from the avitaminosis seem to be a more probable explanation.

Chief *post mortem* findings:

2393. Large tongue abscess; abscess in lung; caecum—large abscess, perforated.
2377. Pyelitis; lungs badly infected.
2593. Lungs full of chronic abscesses.
2351. Large tongue abscess; keratosis of stomach; ballooning of stomach and intestines.
2446. Chronic abscess in lung; chronic ulceration of caecum.
2728. Pyelitis of right kidney; extravasation of urine retroperitoneally.

"appreciable growth" was maintained for 11, 13 and 16 days after dosing began. In the remaining 3 curves the weight response was excellent for even longer periods, *viz.* 24–26 days, after which death occurred suddenly without any "obvious cause," *i.e.* with no outward signs of vitamin A deficiency. A study of the main *post mortem* findings given below the diagram seems to furnish an adequate explanation for the occurrence of weight curves of these types. In our opinion the sudden decline following an initial response is not the result of the complete exhaustion of hitherto undepleted reserves of vitamin A, as suggested by Ahmad and Drummond [1930]. It is due rather to the presence in the animal at the end of the preliminary period of some ineradicable pathological condition, which has increased in severity while the improvement of other conditions has caused a temporary increase in weight. It is to be noted that cases may occur in which the outward appearance of the animal gives no indication of its real condition. Thus rat 2728, which died of advanced pyelitis, was apparently in perfect health 26 days after dosing began and became practically moribund in one night.

(2) *Subnormal growth response.* Among the different types of response to vitamin A dosage tabulated by Hume and Smith [1928] there is included

"recovery with subnormal, or normal, growth for a time, followed by premature flattening." Coward and Key [1928] report similar results and include "continuous subnormal response" as a further type. Bacharach [1933] also speaks of "the frequent failure of the animals, after they have ceased to grow, to respond to even relatively large doses of the vitamin with anything approaching normal growth. It is generally held that this is due to some inadequacy in the basal diet." In Fig. 6 are included a number of curves which may be regarded as illustrative

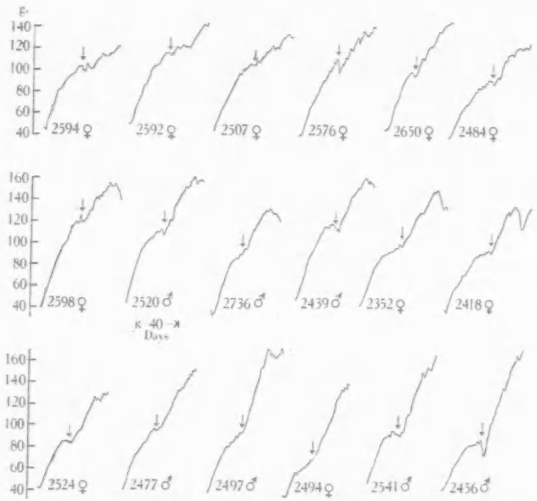



Fig. 6. Persistence of pathological conditions after avitaminosis-A may account for the varying types of response to vitamin A dosage. These graphs show varying degrees of "subnormal response," such as have been described by Hume and Smith, Coward and Bacharach.

Chief *post mortem* findings:

2594. Chronic inflammation of caecum.
2592. Ulceration of caecum.
2507. Chronic inflammation of caecum; perforated ulcer, now closed.
2576. Several healed ulcers in stomach.
2650. Inflammation of lower end of intestine.
2484. Ulceration of caecum.
2598. Pyelitis; small haemorrhagic pits in stomach.
2520. Ulceration of caecum, and patch of chronic inflammation.
2736. Pneumonia.
2439. Large ulcer in caecum.
2352. Keratosis of stomach.
2418. Keratosis of stomach; lymphatics much enlarged; lower end of ileum congested.
2524. Chronic inflammation of caecum; lymphatics enlarged.
2477. Chronic inflammation of caecum.
2497. Chronic inflammation of caecum.
2494. Pyelitis in left kidney.
2541. Inflammation of caecum, and 3 ulcers.
2456. Chronic inflammation of stomach.

of these types. We have found that "continuous subnormal response" is very frequently associated with inflammation or ulceration of the caecum. The premature slackening which may follow a period of subnormal or normal growth is undoubtedly due to the persistence of pathological conditions dating from the

preliminary depletion period. Hume and Smith were inclined to draw this conclusion from their own work but apparently rejected it after a scrutiny of the work of others. "Some inadequacy in the basal diet" seems a less adequate explanation of the failure to attain normal growth than the fact of the persistent pathological conditions arising from the vitamin A deficiency. Even when the animals have responded with practically normal growth performance during the test period, *e.g.* rats 2456, 2541 and 2494, it will be seen that such conditions as inflammation of the stomach, ulceration and inflammation of the caecum and pyelitis may still persist.

We have evidence which shows that the pathological conditions due to vitamin A deficiency may persist even after several months on normal diet. In Table III are given data for 5 rats which, after being used in vitamin A tests,

Table III. *Persistence of pathological conditions due to avitaminosis-A after several months on normal diet.*

| No. of rat | Period on normal diet months | *Post mortem* findings |
|---|---|---|
| 580 | 8½ | Pleurisy; ulceration of stomach; small ulcer in duodenum; peritonitis round caecum |
| 1631 | 3 | Chronic abscess involving most of left lung; areas of consolidation in right lung |
| 1538 | 4 | Chronic abscess in apex of right lung, and consolidated patches in rest of same lung; caecum—perforated ulcer, partially healed |
| 1588 | 4 | Caecum—two chronic ulcers |
| 1706 | 3 | Caecum—perforated ulcer, healed |

had been placed on normal diet for periods varying from 3 to 8½ months. These animals had appeared to be in good condition at the end of the vitamin test, but as they had afterwards failed to attain "normal growth performance" on normal diet, they were killed and examined for macroscopic lesions.

In other two rats examined after 3 and 4½ months on normal diet no definite macroscopic lesions were found, but it is significant that even in these cases, and in that of rat 1706, in which the main lesion was a healed ulcer in the caecum, the growth performance and condition of the animals remained subnormal. It was pointed out by Macy *et al.* [1927, 1] that if the preliminary period were too prolonged, advanced pathological conditions might be established which could not be ameliorated by vitamin adjuvants. They found that generalised infections resulting in the localisation of pus in the upper part of the digestive and respiratory tracts could not be cured by feeding methods, regardless of the richness of the diet in vitamin A. Few assayers, however, seem to have realised the significance of such results.

This persistence of pathological conditions that have been induced by vitamin A deficiency, taken in conjunction with their diverse nature, forms the strongest argument against the curative method of assay. In a curative test vitamin A can influence growth only indirectly, by effecting improvement in the pathological conditions, and it is obvious that no consistency in results can be expected when these conditions may vary so widely not only in their nature but in their intensity and amenability to treatment. It has been shown that with a comparatively short depletion period there is more likelihood of a good response to vitamin A dosage, but even a considerable reduction in the length of this period cannot ensure similarity in the condition of the experimental animals.

*Early occurrence of pathological conditions in vitamin A deficiency.*

The results of the second milk assay, recorded in Table II and Fig. 2, seemed to indicate that varied pathological conditions must have been present in the animals before the weight curve had shown any marked flattening. This point was therefore investigated in a later experiment, in which a number of rats were killed, not in moribund condition, after periods of 3–7 weeks on vitamin A-free diet. The results were as follows:

| | | | | | |
|---|---|---|---|---|---|
| No. of weeks on diet | 3 | 4 | 5 | 6 | 7 |
| No. of rats examined | 19 | 18 | 20 | 8 | 5 |
| No. classed as "normal," *i.e.* with no macroscopic lesions | 3 | 6 | 0 | 0 | 0 |

It will be seen that of 37 rats killed after 3 and 4 weeks, *i.e.* before they would be considered to be "depleted" of vitamin A according to the usual criteria, there were only 9 in which no macroscopic lesions were detected. In rats killed from 5 weeks onwards none were classed as normal. The conditions found included ulceration and haemorrhagic points in the glandular part of the stomach, inflammation of the small intestine, especially the duodenum, and inflammation or ulceration of the caecum. These findings are of particular interest in view of the instructions given in the M.R.C. Report for carrying out a prophylactic assay of vitamin A: "Doses of the material to be tested are given either from the beginning of the feeding with the experimental diet or from about 3 weeks after, when the rats' reserves of vitamin A have been partially exhausted." The evidence given above shows that a test in which dosing is begun after 3 weeks on the basal diet cannot be regarded as prophylactic but is essentially of the curative type.

A close study of the weight curves during the preliminary period of a curative test shows that in many cases divergence from normal begins to show very early. For a rat growing normally this part of the weight curve should be practically a straight line. In Fig. 4, where the earlier course of the weight curve has been continued by means of a dotted line, it will be seen that divergence begins after periods of 8–18 days on the basal diet, except in the case of rat 2565, where the normal course was followed for 26 days. For the 23 examples shown in this figure, the average time of divergence is 13·5 days. It seems very probable therefore that these effects on the weight curve are an indication that diseased conditions had already begun to develop even at this early stage.

*Reserve stores of vitamin A in young rats.*

The early onset of disease in young rats deprived of vitamin A gives rise to serious doubt regarding their supposed reserve store of the vitamin and the time necessary for its depletion. According to Green and Mellanby [1928] there is no doubt that these stores rapidly disappear. They found that even in the case of rats dying at an early stage, before any great pathological change had occurred, liver extracts gave negative results when tested colorimetrically for vitamin A. Dann [1932] showed that the amount of vitamin A in the livers of rats at birth was small and was unaffected by the amount of carotene eaten by the mother before or during pregnancy, although the amount at weaning could be increased by feeding the mother with a diet rich in carotene. It was found, however, that the weight of the young at weaning and their weight and age when growth ceased on a vitamin A-free diet appeared to be independent of the amount of vitamin A in the mother's diet during pregnancy or lactation, except when she was almost depleted of vitamin A. This would appear to indicate that any extra

stores of vitamin A acquired before weaning are rapidly depleted and have no practical effect on the length of the preliminary period. There seems therefore to be little justification for the practice commonly adopted of restricting the mother's intake of vitamin A during breeding, in order that the reserve stores of vitamin in the young rats may be kept low. There is no doubt that when rats so bred are placed on a vitamin A-free diet, their weight curves will tend to flatten at a lower level than those of animals bred on a more liberal system, but no clear proof appears to have been given that this difference is due to differences in the vitamin A reserve stores of the young rats, as is generally assumed [Smith and Chick, 1926]. The possibility has apparently been overlooked that differences in the levels at which the weight curves flatten may be due simply to differences in the vigour and health of the animals. That the system of breeding adopted tends to produce an inferior type of rat is indicated by the falling off in breeding capacity reported by various workers. Thus Smith and Chick [1926] state that litters after the third are small and irregular, and Scheunert and Schieblich [1933] report that they have been unable to maintain a supply of standard young rats on any of the standard diets in the literature. Cramer [1923; 1924] showed how restriction of the vitamin supply in early life may lead to imperfect development in rats without necessarily producing obvious ill-health. Similarly, Macy *et al.* [1927, 2] found that advanced tissue changes characteristic of vitamin A deficiency appeared very early (6–12 days) in rats whose mothers had received inadequate vitamin A, and the evidence indicated the probability that these changes had begun during lactation. There seems therefore to be a serious danger that the approved system of breeding may result in producing young rats which, if not already suffering from incipient vitamin A deficiency, are at least subnormal in regard to their standard of health, and that animals considered "suitable" for vitamin A tests are not merely low in vitamin A reserves but low in vitality.

The facts that macroscopic evidence of disease was found in 75 % of the rats examined after 3–4 weeks on the deficient diet and that the weight curves begin to diverge from normal at as early a time as 13·5 days indicate that any reserve stores of vitamin A in the young rat are depleted much more rapidly than is generally assumed. It is probable that histological examination might reveal evidence of the effects of the deficiency at a still earlier stage. The whole question of the "reserve store" of vitamin A seems to require further investigation. In the case of the young animal the chief argument in favour of the existence of these reserve stores appears to be the work of Korenchevsky and Carr [1924]. These authors found that excess of the fat-soluble factor in the mother's diet during pregnancy caused a marked decrease in the disorders of general nutrition produced in the young by a diet deficient in fat-soluble factor. It does not follow from this, however, that "variation in the state of nutrition of the young rats" is synonymous with "variations in the reserves" of the fat-soluble vitamins possessed by the young animals at the beginning of the experimental period [Smith and Chick, 1926]. If the results of Korenchevsky and Carr are taken in conjunction with the finding of Dann that the vitamin A content of the liver at birth is unaffected by the amount of carotene in the mother's diet during pregnancy, they show rather that an ample supply of the fat-soluble factors is essential in the mother's diet during pregnancy to ensure the production of healthy offspring.

Sherman and Cammack [1926] made a quantitative study of the storage of vitamin A in older animals, of ages 2–6 months, which, after weaning, had been given varying amounts of vitamin A before being placed on the deficient diet.

REFERENCES.

Ahmad and Drummond (1930). *Biochem. J.* **24**, 27.
Bacharach (1933). *Biochem. J.* **27**, 5.
Coward and Key (1928). *Biochem. J.* **22**, 1019.
—— Key, Dyer and Morgan (1930). *Biochem. J.* **24**, 1952.
—— Dyer, Morton and Gaddum (1931). *Biochem. J.* **25**, 1102.
—— Cambden and Lee (1932, 1). *Biochem. J.* **26**, 679.
—— Dyer and Morton (1932, 2). *Biochem. J.* **26**, 1593.
—— Key and Morgan (1933). *Biochem. J.* **27**, 873.
Cramer (1923). *Lancet*, i, 1046.
—— (1924). *Lancet*, i, 633.
Culhane (1933). *Biochem. J.* **27**, 69.
Dann (1932). *Biochem. J.* **26**, 1072.
Drummond, Coward and Handy (1925). *Biochem. J.* **19**, 1068.
—— and Morton (1929). *Biochem. J.* **23**, 785.
Dutcher, Honeywell and Dahle (1927). *J. Biol. Chem.* **75**, 85.
Green and Mellanby (1928). *Brit. Med. J.* ii, 691.
Hume and Smith (1928). *Biochem. J.* **22**, 504.
Korenchevsky and Carr (1924). *Biochem. J.* **18**, 1313.
Macy, Outhouse, Long and Graham (1927, 1). *J. Biol. Chem.* **73**, 153.
—— —— Graham and Long (1927, 2). *J. Biol. Chem.* **73**, 175.
Medical Research Council (1932). *Spec. Rep. Series*, No. 167.
Norris (1931). *Bull. Basic Sci. Res.* **3**, 249.
Orr and Richards (1934). *Biochem. J.* **28**, 1259.
Sampson, Dennison and Korenchevsky (1932). *Biochem. J.* **26**, 1315.
Scheunert and Schieblich (1933). *Biochem. Z.* **263**, 444.
Schmidt-Nielsen (1929). Quoted by Ahmad and Drummond (1930)
Sherman and Burtis (1928). *J. Biol. Chem.* **78**, 671.
—— —— Cammack (1926). *J. Biol. Chem.* **68**, 69.
Smith and Chick (1926). *Biochem. J.* **20**, 131.
Steenbock and Coward (1927). *J. Biol. Chem.* **72**, 765.
Wise and Heyl (1932). *J. Amer. Pharm. Assoc.* **21**, 1142.

# CLXXIV. THE DETERMINATION OF CYSTINE. USE OF THE ZEISS PHOTOMETER.

By JOHN HERBERT BUSHILL, LESLIE HERBERT LAMPITT and LESLIE CHARLES BAKER.

*From the Laboratories of Messrs J. Lyons and Co., Ltd., London, W.14.*

(*Received May 19th, 1934.*)

Most available methods for the determination of cystine depend upon its reduction to cysteine which in turn reduces a reagent with the production of a coloured solution. The methods suffer from the disadvantage, more or less general to all colorimetric reactions, that the accuracy is liable to be vitiated by factors only partly understood, since the conditions of the production of the colour have been defined in an empirical manner.

Presumably the claims for specificity made by Sullivan [1926] for his method have resulted in the adoption of his technique in much work reported since 1926, but when some years ago (1927) we applied this method to hydrolysed proteins, its unreliability became at once apparent. Rimington, in a private communication [1929], confirmed this unreliability, and the question has since been studied by Lugg [1933] who has modified the Sullivan technique by buffering "standard" and "unknown" with glycine, thus controlling the hydrogen ion concentration of the resulting mixed solutions.

The work described in the present paper was undertaken in order to study the accuracy of the results obtained by the Sullivan method and by the modification of Lugg by means of the Pulfrich photometer, as it was thought that such an approach was obviously suitable to the study of a colour reaction[1]. The advantage of the method is that the intensity of the colour can be expressed in absolute units as an extinction coefficient, thus enabling the rate of colour development, the stability and reproducibility of the colour formed and the range and accuracy of the proportionality between colour and cystine to be determined.

## Experimental.

### *The photometer method.*

The light transmission of the substance in solution is compared with that of the solvent in cells of the same thickness, and the formula for the calculation of extinction coefficient $E = \log (I_0/I)/d$ then becomes

$$E = K \frac{(\log I_0 - \log I)}{C},$$

where $I_0$ = intensity of incident light,
$I$ = intensity of emergent light,
$C$ = concentration of substance in solution,
$K$ = a constant,
$E$ = extinction coefficient.

[1] It may be remarked that other workers have had the same idea, for apparently concurrently with this work, Krijgsman and Bouman [1933] applied the photometer to the determination of cystine in blood.

It is evident that the term "solvent" includes all the substances in the system under examination with the exception of the component to be determined, a fact which is only of importance when such substances absorb light of the wave-length employed.

*The choice of wave-length (colour filters).*

In the first instance it was necessary to determine the wave-length of light absorbed by the coloured substance produced from cystine, and whether such light is absorbed by the reagents employed in the development of that colour.

It is important to note that in the development of the cystine-quinone colour the reagent solutions must be freshly prepared. In the case of the sodium $\beta$-naphthoquinonesulphonate this was emphasised by Folin [1922] in his original paper, but not by Sullivan [1926], while Rimington in the above-mentioned private communication [1929] and Prunty [1933] indicated the necessity of using freshly prepared hydrosulphite solution.

The colour was developed according to Sullivan's technique in a series of solutions containing 30 to 210 parts of cystine per million, and the extinction coefficients were determined using light (obtained by the aid of light filters fitted to the photometer) of wave-lengths varying from 430 to 720 $m\mu$.

Consideration of the results obtained (Fig. 1) shows that the widest and most even spacing of the curves occurs when wave-lengths 470, 500 and 530 $m\mu$

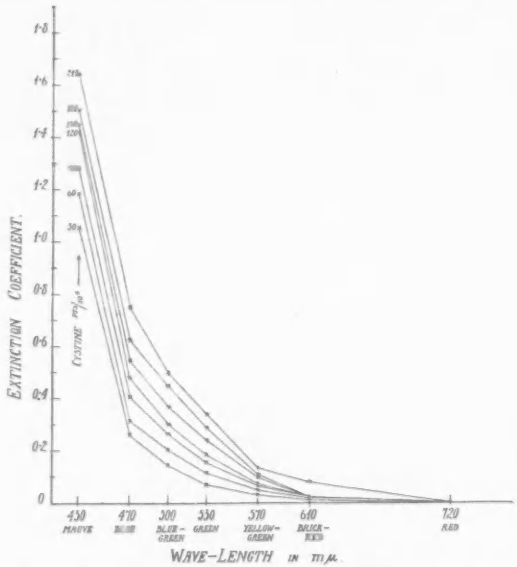



Fig. 1. *Sullivan technique.* The relationship between extinction coefficient and wave-length with varying concentrations of cystine.

are employed (light filters Nos. 47, 50 and 53 respectively). These three wave-lengths were therefore chosen for use in the initial experiments, from the results of which light of wave-length 530 $m\mu$ (filter No. 53) was finally shown to give extinction coefficients proportional to the amount of cystine present.

*The reproducibility of the colour and the influence of the reagents on its determination.*

Sullivan and Lugg add different volumes of reagents to the cystine solution to develop the colour. For this reason in these experiments standard cystine solutions[1] were used such that the final concentration of cystine should be approximately the same in the two methods. Table I shows that this is so when a standard cystine solution of 150 parts per million is used in the Sullivan method and one of 600 parts per million in the Lugg method.

Table I.

| | Standard cystine sol. | | Final vol. of coloured solution ml. | Conc. of cystine in coloured solutions (parts per million) |
|---|---|---|---|---|
| | Vol. used ml. | Conc. parts per million | | |
| Sullivan's method | 5 | 150 | 16 | 47 |
| Lugg's method | 2 | 600 | 25 | 48 |

A large number of determinations was made of the colour developed by the two methods in their respective standard solutions (Table I) by measuring the extinction coefficients at the three chosen wave-lengths. Since it was observed that the reagents alone produce a yellow colour (more intense in the case of Lugg's method) the extinction coefficients of this colour (the "blank") were determined in a similar manner.

The *average extinction coefficients* recorded in Table II are calculated from a series of determinations, usually six in number, while the figures recorded as *variations in extinction coefficient* are the maximum differences from the average figure for the series.

This maximum difference is calculated as a percentage of the average extinction coefficient and recorded as *percentage variations in extinction coefficient*. The "modified Lugg method" quoted in this table is described later.

The percentage variations in extinction coefficient in Table II demonstrate the decided superiority of the modified Lugg method over that of Sullivan.

The average extinction coefficients of the reagents alone show that they absorb a small amount of light of wave-lengths 470, 500 and 530 $m\mu$ and will therefore affect to a small extent a cystine determination. Since this "reagent colour" must be present in all determinations of cystine, it is clearly desirable to use a reagent "blank," instead of water, against which the light transmission of the colour developed by cystine is matched.

*The effect of atmospheric oxygen on colour development.*

While studying the rate of colour development in a cystine solution and the stability of that colour, it was noticed that if the coloured solution were transferred from a flask to the matching cell and left undisturbed (protected from the air by a thin glass plate) the intensity of the colour reached a maximum in about 10–20 minutes and then faded very slowly. When, on the other hand, the solution was poured backwards and forwards from the cell to the flask for each light measurement to be taken a progressive darkening of the solution

[1] The cystine employed in all experiments described herein was 95 % pure by nitrogen determination.

Table II. *Extinction coefficients of coloured solutions matched against water.*

| Method | Reagents alone: Average extinction coefficient (wave-lengths) 470 mμ | 500 mμ | 530 mμ | Variation in extinction coefficient 470 mμ + | 470 mμ − | 500 mμ + | 500 mμ − | 530 mμ + | 530 mμ − |
|---|---|---|---|---|---|---|---|---|---|
| Sullivan (1) | — | — | — | — | — | — | — | — | — |
| ,, (2) | — | — | — | — | — | — | — | — | — |
| ,, (3) | 0·139 | 0·064 | 0·049 | 0·066 | 0·047 | 0·040 | 0·034 | 0·032 | 0·027 |
| ,, (4) | 0·114 | 0·048 | 0·045 | 0·029 | 0·015 | 0·017 | 0·008 | 0·016 | 0·014 |
| Lugg | — | — | — | — | — | — | — | — | — |
| Lugg modified (1) | 0·298 | 0·093 | 0·029 | 0·017 | 0·008 | 0·009 | 0·005 | 0·009 | 0·005 |
| ,, (2) | 0·291 | 0·091 | 0·029 | 0·025 | 0·014 | 0·030 | 0·015 | 0·023 | 0·007 |
| ,, (3) | 0·294 | 0·087 | 0·025 | 0·010 | 0·007 | 0·006 | 0·003 | 0·005 | 0·004 |

| Method | Reagents + 47–48 pts./$10^6$ of cystine: Average extinction coefficient (wave-lengths) 470 mμ | 500 mμ | 530 mμ | Variation in extinction coefficient 470 mμ + | 470 mμ − | 500 mμ + | 500 mμ − | 530 mμ + | 530 mμ − | Percentage variation in extinction coefficient 470 mμ + | 470 mμ − | 500 mμ + | 500 mμ − | 530 mμ + | 530 mμ − |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sullivan (1) | 0·626 | 0·667 | 0·559 | 0·097 | 0·125 | 0·107 | 0·124 | 0·079 | 0·075 | 15·5 | 20·0 | 16·0 | 18·6 | 14·1 | 13·6 |
| ,, (2) | 0·642 | 0·687 | 0·537 | 0·097 | 0·074 | 0·153 | 0·099 | 0·134 | 0·068 | 15·1 | 11·5 | 22·3 | 14·4 | 25·0 | 12·7 |
| ,, (3) | 0·841 | 0·945 | 0·793 | 0·106 | 0·049 | 0·127 | 0·105 | 0·101 | 0·086 | 12·6 | 5·8 | 13·5 | 11·2 | 12·8 | 10·9 |
| ,, (4) | 0·839 | 0·900 | 0·754 | 0·033 | 0·081 | 0·042 | 0·093 | 0·057 | 0·057 | 3·9 | 9·6 | 4·7 | 10·3 | 7·6 | 7·6 |
| Lugg | 0·574 | 0·422 | 0·345 | 0·045 | 0·047 | 0·066 | 0·046 | 0·052 | 0·040 | 7·9 | 8·3 | 15·6 | 10·1 | 15·3 | 11·8 |
| Lugg modified (1) | 0·731 | 0·653 | 0·497 | 0·039 | 0·032 | 0·021 | 0·051 | 0·018 | 0·034 | 5·4 | 4·3 | 3·3 | 7·9 | 3·6 | 6·8 |
| ,, (2) | 0·753 | 0·668 | 0·513 | 0·017 | 0·030 | 0·045 | 0·031 | 0·017 | 0·025 | 2·3 | 4·0 | 6·7 | 4·2 | 3·4 | 5·0 |
| ,, (3) | 0·757 | 0·677 | 0·513 | 0·013 | 0·013 | 0·030 | 0·021 | 0·027 | 0·017 | 1·8 | 1·8 | 4·4 | 3·1 | 5·3 | 3·5 |

occurred. This observation suggested that the darkening was due to the oxygen of the atmosphere.

If this is the case, an excess of the reducing substance, the alkaline sodium hydrosulphite, might retard the oxidation of the reactive substances in the system by preferentially absorbing any atmospheric oxygen, thus delaying the darkening effect.

In an experiment the colour was developed in one portion of a standard cystine solution according to Lugg's technique and in another using the same method but with a concentration of sodium hydrosulphite three times the usual strength. A portion of each coloured solution was transferred to a matching cell and left undisturbed, while the remainder of each solution was shaken periodically. The results of determinations of the extinction coefficients obtained at all three wave-lengths were similar; those at wave-length 530 $m\mu$ are shown in Fig. 2.

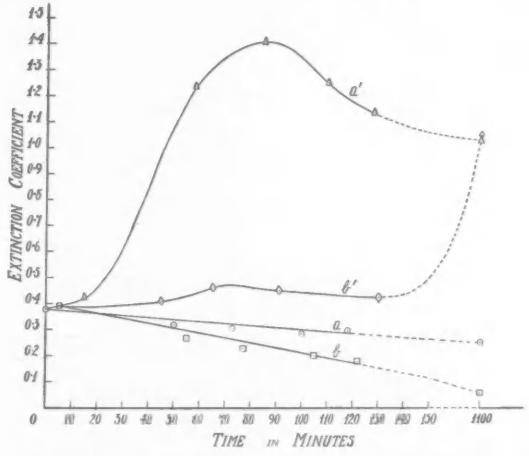



Fig. 2. *Lugg technique.* The relationship between extinction coefficient and time with and without aeration.

| | | | |
|---|---|---|---|
| *a* | Standard amount of hydrosulphite | | —No shaking |
| *a'* | ,, | ,, | —Shaken |
| *b* 3× | ,, | ,, | —No shaking |
| *b'* 3× | ,, | ,, | —Shaken |

It will be noted that a slow fading takes place in the solutions not shaken with air while if the solution containing the normal amount of hydrosulphite is shaken it darkens rapidly. This change is dramatic and may be observed by eye. Where three times the usual concentration of hydrosulphite was used the darkening was delayed.

Similar experiments have been made employing the Sullivan method of colour development. The results which are shown in Fig. 3 indicate that changes in extinction coefficient do occur with and without shaking but that, in distinction from the results when the Lugg technique was employed, such changes vary with different wave-lengths.

These results appear to be particularly interesting but it is not proposed at the moment to pursue the matter further. Suffice it to say that the colour developed by the methods both of Sullivan and of Lugg is affected by shaking in the presence of air.

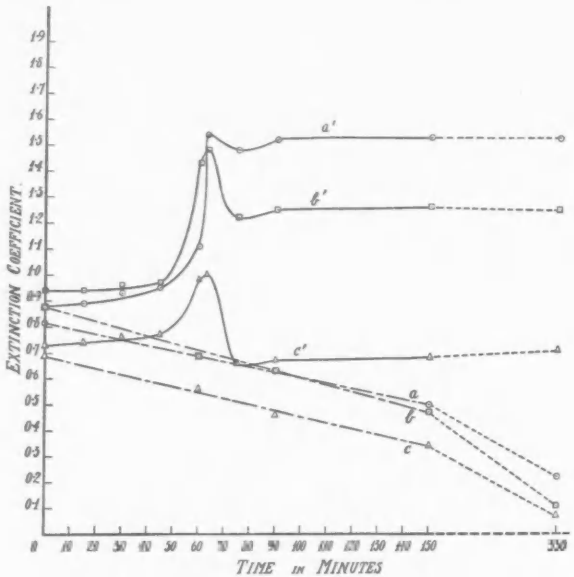



Fig. 3. *Sullivan technique.* The relationship between extinction coefficient and time with and without aeration using light of different wave-lengths.

| | | | | | |
|---|---|---|---|---|---|
| *a* | Solution not shaken— | | light | wave-length | 470 mμ |
| *b* | ,, | ,, | — | ,, | 500 mμ |
| *c* | ,, | ,, | — | ,, | 530 mμ |
| *a'* | ,, | shaken | — | ,, | 470 mμ |
| *b'* | ,, | ,, | — | ,, | 500 mμ |
| *c'* | ,, | ,, | — | ,, | 530 mμ |

*The determination of cystine in pure solution based on its relationship with the colour developed by the modified Lugg technique.*

Lugg developed the colour from cystine solutions in 25 ml. stoppered cylinders. It is obviously advantageous to use 25 ml. graduated flasks in order that the coloured solution may be accurately diluted to the mark with water after the addition of hydrosulphite, rather than to rely on about ten pipette measurements to aggregate 25 ml.

The slight modifications of the Lugg technique employed in this work are as follows:

In order to eliminate the effect of atmospheric oxygen (already demonstrated) the column of air above the liquid in the 25 ml. flask was displaced by passing in a rapid stream of purified nitrogen for half a minute immediately before and after the addition of the sodium hydrosulphite reagent. The quinone, sodium sulphite and sodium hydrosulphite solutions were freshly prepared,

the last in an atmosphere of purified nitrogen. The reagents were stored and mixed in flasks held in a water-bath maintained at 21°.

A series of solutions containing 240–960 parts per million of cystine in 0·1 *N* hydrochloric acid were prepared, the solution containing 600 parts per million being regarded as the "standard" and the others as "unknowns." Colour was developed simultaneously in each "unknown" cystine solution, in the "standard" and in the "blank" (reagents alone).

With each set of three solutions corresponding to each concentration of cystine, extinction coefficients were obtained by matching both the "standard" and the "unknown" against the blank (using matching cells 5 mm. thick).

The results are shown in Fig. 4 where mean straight lines have been drawn through the experimental points. Since the colour was developed in the standard (600 parts per million of cystine) at the same time as in each of the unknowns,

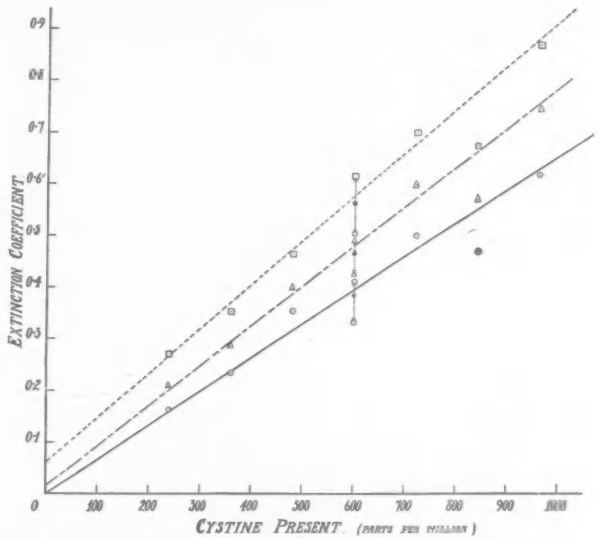



Fig. 4. The relationship between cystine content and extinction coefficient (matching against reagent "blank").

⊙———⊙ 470 $m\mu$ ⊡- - - -⊡ 500 $m\mu$ ◬— — —◬ 530 $m\mu$

several such standards (six in all) were prepared and examined. The average values for the extinction coefficients of the standards are shown in the above-mentioned figure as solid points, while the range is shown by the vertical lines.

Fig. 4 shows that when using this technique there is an approximately linear relationship between the cystine present and the extinction coefficient of the coloured solution obtained therefrom.

It is evident that the proximity of the line to the origin indicates with what success the colour developed by the cystine alone has been determined. It follows therefore that the results shown in Fig. 4 indicate that wave-lengths 530 and 470 are preferable to 500 $m\mu$.

When the colours are matched against "blank reagents" the interfering colours produced in the solutions are largely eliminated thus enabling reasonable results to be obtained at all three wave-lengths (Fig. 4). When however matching is made against "water," instead of the blank reagents, the wave-lengths at which "reagent colours" are also determined become evident by showing less proportionality between extinction coefficient and the cystine present. Such esults were obtained from the solutions of the above experiment and are shown in Fig. 5 where it is evident that at wave-length 470 $m\mu$ colours not derived from cystine interfere.

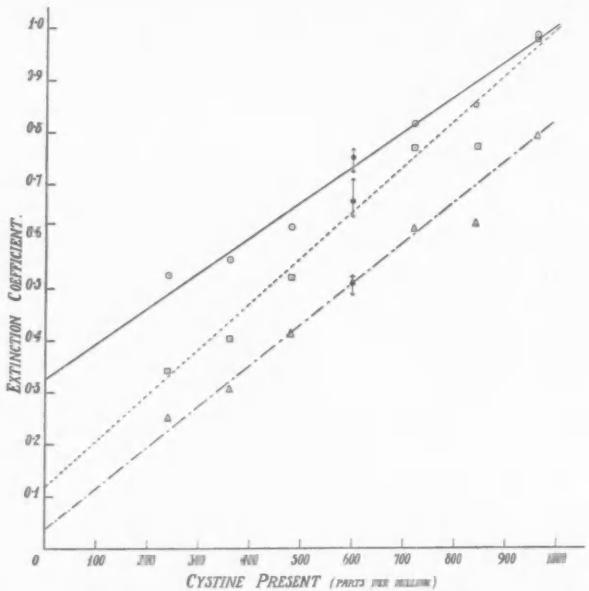



Fig. 5. The relationship between cystine content and extinction coefficient (matching against water).

⊙———⊙ 470 $m\mu$ ⊡- - - - -⊡ 500 $m\mu$ △— — —△ 530 $m\mu$

Examination of Figs. 4 and 5 therefore enables the conclusion to be drawn that when employing light of wave-length 530 $m\mu$ there is best proportionality between the determined extinction coefficient of the solution and the cystine present. This, therefore, is the wave-length 530 $m\mu$ (filter No. 53) which should be employed in the determination of cystine, and at which matching against the blank reagents should, for reasons already stated, be employed.

It follows that cystine may be determined in an "unknown" by simple proportion from the determined extinction coefficient of the coloured solution (prepared by the modified Lugg technique) and that of a standard cystine solution prepared at the same time.

When such calculations are made from the results recorded in Fig. 4, employing as the "standard" the 600 parts per million cystine solution in which the colour was developed at the same time as in the "unknown," the results

shown in Fig. 6 were obtained. The percentage errors of the determinations are calculated from the results plotted in Fig. 4 and are shown in Table III A. These errors are obtained by expressing the deviations of the individual points from the mean straight line as a percentage of cystine present.

It will be noted from Figs. 4 and 6 that occasionally, and for no apparent reason, an abnormal result may be obtained (*e.g.* 840 pts./$10^6$, Fig. 4; 720 pts./$10^6$, Fig. 6). It therefore follows that there are disturbing factors other than that of oxidation at present incompletely controlled.

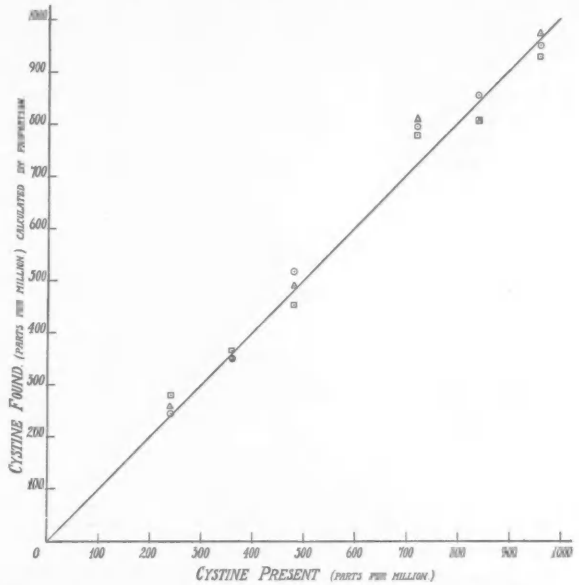



Fig. 6. Relationship between actual and calculated cystine content (from Fig. 4).
⊙ 470 $m\mu$ ⊡ 500 $m\mu$ ◬ 530 $m\mu$

*The determination of cystine in the presence of hydrolysed caseinogen based on its relationship with the colour developed by the modified Lugg technique.*

A series of solutions each containing 10 % hydrolysed caseinogen (Hammarsten's casein) and different amounts of added cystine was prepared. Using the modified Lugg technique, the colour was developed in each of these solutions, and the extinction coefficients were determined.

The results are plotted in Fig. 7 (matched against the blank reagents). It is evident that the experimental points fall more nearly on a straight line than did those obtained using pure cystine. This is perhaps more readily demonstrated by the percentage errors shown in Table III B, where it is shown that the error is approximately $\pm 8$ %, using a cystine concentration greater than 200 pts./$10^6$.

Fig. 7 also demonstrates that a cystine determination employing wave-length 570 $m\mu$ would, owing to the slope of the curve, be much less accurate than one employing 530 $m\mu$ wave-length; the former was the wave-length of

Table III.

| A Estimation of cystine in pure solution | | B Estimation of cystine in the presence of hydrolysed caseinogen | | | | C Estimation of cysteine in pure solution | | |
|---|---|---|---|---|---|---|---|---|
| Cystine present pts./$10^6$ | Percentage error | Cystine equivalent of hydrolysed caseinogen solution pts./$10^6$ | Cystine added pts./$10^6$ | Total cystine present pts./$10^6$ | Percentage error | Cysteine present pts./$10^6$ | Percentage error | |
| | | | | | | | Exp. I | Exp. II |
| 240 | + 5·8 | 100 | 0 | 100 | −12·0 | 60 | 0·0 | +11·6 |
| 360 | 0·0 | 100 | 100 | 200 | +15·0 | 120 | −4·2 | + 6·7 |
| 480 | + 4·7 | 100 | 200 | 300 | 0·0 | 180 | −1·7 | − 4·2 |
| 600 | max. + 3·3 | 100 | 300 | 400 | + 8·2 | 240 | +0·8 | + 4·2 |
| | av. − 2·1 | 100 | 400 | 500 | + 7·4 | 300 | 0·0 | − 1·7 |
| | min. −10·3 | 100 | 500 | 600 | + 1·2 | 300 | +1·0 | 0·0 |
| 720 | + 4·9 | 100 | 600 | 700 | − 5·0 | 360 | −3·5 | + 0·8 |
| 840 | −13·7 | 100 | 700 | 800 | − 7·5 | 420 | −2·6 | + 3·3 |
| 960 | − 1·2 | 100 | 900 | 1000 | + 3·0 | 480 | +0·4 | + 1·3 |
| | | | | | | 540 | +0·2 | − 1·3 |

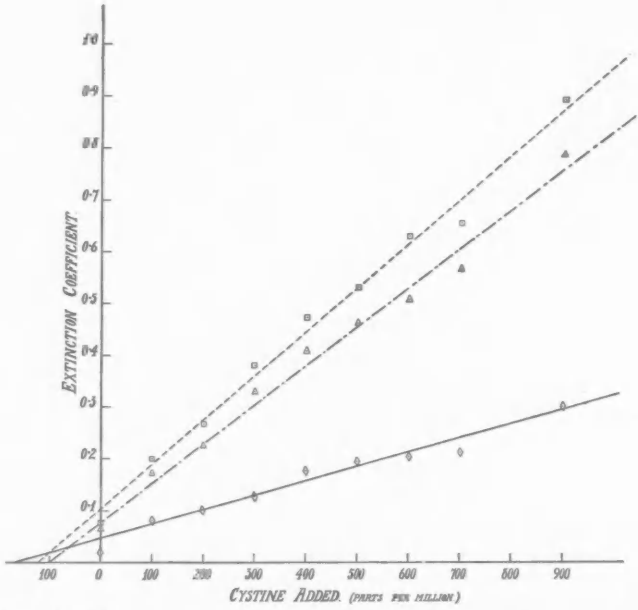



Fig. 7. The relationship between extinction coefficient at various wave-lengths and cystine in the presence of hydrolysed caseinogen.

⊡------⊡ 500 $m\mu$ ◬—·—·—◬ 530 $m\mu$ ◇————◇ 570 $m\mu$

the light chosen by Krijgsman and Bouman for cystine determination (see footnote p. 1293).

It may be mentioned that since extinction coefficients determined at wave-length 530 $m\mu$ are directly proportional to the cystine present, by producing back that line (Fig. 7) until it cuts the abscissa, a measure of the cystine present

in the hydrolysed caseinogen solution used is obtained. This result, the cystine equivalent of the hydrolysed caseinogen solution, is shown in Table III B (*i.e.* 100 parts per million). The amount indicated by this curve does not represent the cystine in caseinogen since during the hydrolysis no precautions were taken to prevent destruction of that amino-acid.

Comparison of Figs. 4 and 7 and also of the results given in Table III A and B indicates that more consistent results are obtained in the presence than in the absence of hydrolysed caseinogen. Lugg has already stated that $p_H$ is an important factor in the colour development, and it is suggested that the improved results in the presence of hydrolysed caseinogen are due to its added buffer capacity which would reduce errors in the adjustment of the $p_H$ during the development of the colour.

One other factor which is believed to be an important one, and which may not be adequately controlled in the modified Lugg method, is the reduction of cystine to cysteine. If such a factor exists then curves constructed similarly to those of Fig. 4 employing cysteine instead of cystine should show an improved straight line relationship.

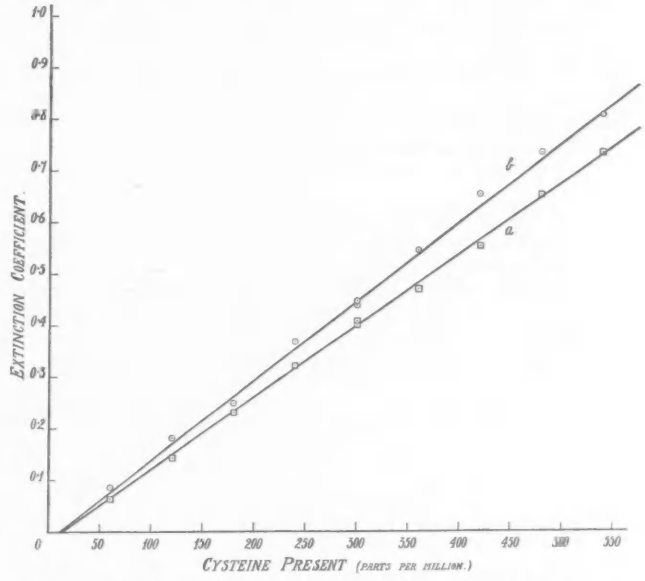



Fig. 8. The relationship between extinction coefficient and cysteine present.
*a*. Exp. 1. *b*. Exp. 2.

*The relationship between the cysteine present and the colour developed by the modified Lugg technique.*

A series of solutions containing 60 to 530 pts./$10^6$ cysteine was prepared and the colours developed from them were matched against a "blank" using light of wave-length 530 $m\mu$.

The results are plotted in Fig. 8, from which the percentage errors have been calculated as previously described and recorded in Table III C, where it will be noted that above a concentration of 120 pts./$10^6$ of cysteine the error is approximately $\pm 4$ %.

It is evident that there is a considerable improvement in the accuracy of the determination of cysteine over that of cystine (Fig. 4, Table III A and B), from which it is concluded that there is a variable factor concerned with the reduction of the cystine in the modified Lugg method.

## Summary.

1. The criticisms of the Sullivan method by Rimington [1929] and by Lugg [1933] have been confirmed by the aid of photometric absolute measurements of the colour produced.

2. Photometric determinations of extinction coefficients have shown that the best proportionality between them and the cystine content is obtained when light of wave-length 530$m\mu$ is used (filter No. 53).

3. The colour produced by the reagents, particularly those of Lugg, is affected appreciably by the presence of air (oxygen). When the colour is developed by the Sullivan method from a cystine solution the extinction coefficient of that solution varies up to $\pm 25$ % from the mean value; by employing the Lugg technique with suitable precautions against oxidation that variation is reduced to $\pm 8$ %.

4. This modified Lugg technique enables cystine in pure solution to be determined with an error of approximately $\pm 5$ %, but with an occasional error of $\pm 15$ %, indicating it is believed, that some factor or factors are not sufficiently controlled.

5. In the presence of hydrolysed caseinogen, when the solution is more highly buffered, the outside limit of error of cystine determination becomes $\pm 8$ %.

6. An indication has been given of a method of determining cystine in hydrolysed caseinogen and presumably in any hydrolysed protein.

7. Cysteine in pure solution may be determined with an error of only $\pm 4$ %, thus indicating that one other factor affecting cystine determination is introduced by the process by which the cystine is reduced to cysteine.

Our thanks are due to Messrs J. Lyons and Co., Ltd., in whose laboratories this work was conducted, for permission to publish this paper.

## REFERENCES.

Folin (1922). *J. Biol. Chem.* **51**, 377.
Krijgsman and Bouman (1933). *Arch. Néerl. Phys.* **18**, 458.
Lugg (1933). *Biochem. J.* **27**, 668.
Prunty (1933). *Biochem. J.* **27**, 387.
Rimington (1929). Private communication.
Sullivan (1926). *U.S. Pub. Health Reports*, No. 1084.

# CLXXV. DIALYSIS OF MILK.
## II. SOME FACTORS AFFECTING THE DISTRIBUTION OF CALCIUM AND PHOSPHORUS.

By LESLIE HERBERT LAMPITT and JOHN HERBERT BUSHILL.

*From the Laboratories of Messrs J. Lyons and Co., Ltd., London, W.14.*

(*Received June 25th, 1934.*)

In a previous paper [Lampitt and Bushill, 1933] reference was made to the unsatisfactory state of knowledge regarding the distribution of the phosphorus of milk as indicated by dialysis, and certain results bearing on the subject obtained by a well-defined technique were recorded. Figures available in the literature for the amount of calcium dialysable from milk are equally unsatisfactory as will be seen from Table I where it is demonstrated that various workers have found as little as 13 % and as much as 50 % of the total calcium in a dialysable form. Much has been written by experimenters on the results obtained by them[1] but it is apparent that before any conclusions can be drawn from dialysis studies, consideration must be given to the factors which may be responsible for the lack of concordance indicated.

As stated in the previous paper, it was considered that certain advantages would accrue from the use of "dried milk" for dialysis experiments, since, by

Table I.

| Reference | Method of preparation of serum: Dialysis | Method of preparation of serum: Filtration | Serum-Ca as % of total Ca in milk |
|---|---|---|---|
| Bell [1925] | — | Chamberland filter | 23·8 |
| Grosser [1913] | — | ,, | 23·4 |
| Magee and Harvey [1926] | Against cold running water, using collodion thimbles | — | 26·4 |
| Mattick [1929] | 25 ml. milk (parchment capsules). 150 ml. 3 % NaCl. Time 18 hrs. Temp. not stated | — | 24–30 |
| Piettre and Achard [1931] | Against water. Temp. not stated | — | 13–25 |
| ,, | Against glycerol. Temp. not stated | — | 29–33 |
| Rona and Michaelis [1909] | 25 ml. water (sac). 1000 ml. milk. Time 2 days. Preservative toluene | — | 40–50 |
| Rossi [1930] | 20 ml. whole milk (collodion sac). 25 ml. water. Time 84 hrs. Temp. "ice-chamber" | — | 40 |
| Van Slyke and Bosworth [1915] | — | Pasteur-Chamberland filter | 22–46 |
| Van Slyke and Bosworth [1916] | — | ,, | 27 |
| Wardlaw [1914] | 25 ml. water (collodion sac). 1000 ml. milk. Time 2 days. Preservative toluene | — | 30–40 |
| Wha [1924] | 10 ml. milk (collodion or parchment sac). 5 ml. water. Time 24 hrs. Temp. not stated. Preservative toluene | — | 50 |

[1] A summary of the available knowledge has been published by Allen [1931].

this means, repetitive experiments could be carried out enabling the technique to be standardised and simplified. Ultimately such technique could form the basis of similar dialysis studies on fresh milk.

In parenthesis it may be remarked that many of the methods used by previous workers for the determination of calcium and of phosphorus in milk and in serum have been found not to be absolutely reliable.

As a means of standardising technique, the use of dried milk still appears to be sound in principle provided that the milk powder used is a standard product and does not differ to any large extent in its chemical and physical attributes from the original milk from which it was produced. The dried milk[1] used in all this work has been of such a character; it represents mixed bulk separated milk chiefly from Red Devon and from Shorthorn herds and "morning" and "evening" milk taken indiscriminately and at various seasons of the year. Justification for the claim that such a product represents "average" milk, certainly as concerns the constituents studied herein, is afforded by the fact that the phosphorus and calcium contents, calculated on non-fatty solids, of a considerable number of different samples fall within a comparatively narrow range. This is illustrated by the following results:

| | | | |
|---|---|---|---|
| Total P | ... | ... | 0·98–1·14 % (mostly 0·98–1·07 %) |
| Total Ca | ... | ... | 1·31–1·44 % |

It is difficult to compare these figures with those of other workers owing to the unknown composition of the milks used by them, but assuming a total non-fatty solids content of 9 %, the following figures are typical:

| | | % |
|---|---|---|
| Total P. | Van Slyke and Bosworth [1915, 1916] ... | 0·95–1·40 |
| | Jordan *et al.* [1906] ... ... ... | 0·85–1·20 |
| | Lenstrup [1926] ... ... ... ... | 1·00–1·10 |
| Total Ca. | Van Slyke and Bosworth [1916] ... | 1·36–2·43 |
| | Mattick [1929] ... ... ... ... | 1·29–1·49 |

The dried milk in question has a solubility greater than 99·8 % when tested by the method previously described [Lampitt and Hughes, 1924; Lampitt and Bushill, 1931], and moreover, as far as can be determined by ordinary analysis, its "solution" is practically indistinguishable from the milk from which it was made. However, the results obtained from the work on phosphorus indicated that possibly the heat treatment to which the product had been subjected had effected a change in the ratio of dialysable to non-dialysable phosphorus; other workers have reported a similar effect both with phosphorus and calcium. Some preliminary work on this point is reported herein, and two other factors have also been studied, namely the effect of the "acidity" and of the dilution of the milk. It is not necessary to emphasise the importance of the effect of "acidity" on milk, particularly in respect of those constituents containing calcium; this factor has been studied by dialysis of milk after inoculation with an acid-producing organism.

The difference between the results obtained with the "continuous" and with the "static" methods of dialysis, which have already been reported, may be attributed to the shifting of the phosphorus equilibrium by the infinite dilution produced by the first method. Consequently in the work now reported the "static" method only has been employed, so that the results obtained represent most nearly the state of the constituents in the liquid dialysed[2].

[1] The milk powder used in this work was prepared by the Milkal Process.

[2] In these experiments pasteurised milk has been almost exclusively employed, so that they are not affected by the presence of milk-phosphatase [Kay and Graham, 1933].

## Experimental.

### (*a*) *Method of dialysis.*

This was essentially the same as previously detailed [1933]. A collodion sac containing 25 ml. of distilled water is suspended in 900 ml. of the milk under investigation and the whole maintained at 0–5° for a period of 2 days. Tests have shown that by that time the dialysable constituents have come to equilibrium with the liquid in the sac in agreement with the results of Wardlaw [1914], and with the aid of an analysis of the contents of the sac the percentage of the various constituents may be calculated on the non-fatty solids of the original milk. In the results given later the amounts of these dialysable constituents are also given as percentages of the total amount of each constituent originally present in the milk itself.

### (*b*) *Methods of analysis.*

*Calcium.* The method employed for the determination of calcium was a combination of the oxalate precipitation of Washburn and Shearer [1932] with a titration of the calcium oxide obtained therefrom as described by Fiske and Logan [1931]. The precipitation is carried out at $p_H$ 3 to avoid precipitation of phosphate with the calcium oxalate.

The sample is reduced to ash in a platinum dish, the ash dissolved in *N*/2 HCl, washed into a beaker and the volume made approximately to 100 ml. Thymol blue is added and more *N*/2 HCl, if required, to give a pink solution, then 5 ml. of 6·4 % oxalic acid solution are added, and the solution is heated to boiling. *N* $NH_4OH$ is then added, drop by drop, with stirring until the solution is definitely yellow ($p_H$ 3). After heating on a water-bath for 3 hours the solution is cooled, the precipitate collected on a No. 41 Whatman filter-paper and washed with a solution prepared as follows: 5 ml. of 6·4 % oxalic acid solution are diluted to 100 ml. and then adjusted by the addition of $NH_4OH$ to $p_H$ 3—yellow to thymol blue. The filter-paper and washed precipitate are reduced to an ash in a platinum dish by heating with a Bunsen burner followed by an hour's treatment at 1000° in a gas muffle. The CaO so produced is dissolved in 5 ml. of *N*/2 HCl, washed into a conical flask with cold boiled water and the excess acid titrated (micro-burette) with *N*/2 NaOH, using methyl red as indicator. With a titration of 3 ml. the error is less than 2 %.

*Phosphorus.* The total phosphorus and the inorganic phosphorus were determined by the method given in the earlier paper [1933] (the colorimetric method of Kuttner and Lichtenstein [1930] being employed), the error of the P determination being less than 2 %.

*Acidity and* $p_H$. The titratable acidity was determined by titration with *N*/10 NaOH (phenolphthalein as indicator) and the result calculated as lactic acid %. It is of course realised that this is an absolutely arbitrary method of expression, but it is simple and convenient as indicating change during the development of acid. The $p_H$ was determined by means of the glass electrode described by Kerridge [1926] employing the potentiometric valve circuit of Morton and Best [1933].

### (*c*) *The dilution effect.*

Dialyses were carried out on separated milk, on the powder (brought previously to a total solids content of approximately 9 %) obtained by spray-drying the same milk, and on these milks or "solutions" diluted with twice their volume of water. The results obtained from the analysis of the dialysates are shown in Tables II and III.

Table II.

Figures represent percentages.

| | Pasteurised milk | | | Solution of powder (1) | | |
|---|---|---|---|---|---|---|
| | | Dialysate | | | Dialysate | |
| | Original milk | Undiluted milk | Diluted milk | Original milk | Undiluted milk | Diluted milk |
| Calculated on non-fatty solids: | | | | | | |
| Ca | 1·34 | 0·41 | 0·58 | 1·36 | 0·44 | — |
| Inorganic P | 0·63 | 0·40 | 0·46 | 0·66 | 0·38 | 0·44 |
| Organic P (by diff.) | 0·35 | 0·10 | 0·10 | 0·33 | 0·08 | 0·06 |
| Calculated on total present in milk: | | | | | | |
| Ca | 100 | 31 | 43 | 100 | 33 | — |
| Inorganic P | 65 | 40 | 47 | 67 | 39 | 44 |
| Organic P (by diff.) | 35 | 10 | 10 | 33 | 8 | 6 |

Table III.

Figures represent percentages.

| | Powder (2) | | | Powder (3) | | | Powder (4) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Dialysate | | | Dialysate | | | Dialysate | |
| | Original milk | Undiluted milk | Diluted milk | Original milk | Undiluted milk | Diluted milk | Original milk | Undiluted milk | Diluted milk |
| Calculated on non-fatty solids: | | | | | | | | | |
| Ca | 1·43 | 0·36 | 0·43 | 1·31 | 0·38 | 0·49 | 1·35 | 0·43 | 0·52 |
| Inorganic P | 0·71 | 0·35 | 0·41 | 0·69 | 0·35 | 0·41 | 0·68 | 0·35 | 0·42 |
| Organic P (by diff.) | 0·35 | 0·10 | — | 0·30 | 0·10 | 0·10 | 0·26 | 0·10 | — |
| Calculated on total present in milk: | | | | | | | | | |
| Ca | 100 | 25 | 30 | 100 | 29 | 37 | 100 | 32 | 39 |
| Inorganic P | 67 | 33 | 39 | 69 | 35 | 41 | 73 | 37 | 45 |
| Organic P (by diff.) | 33 | 10 | — | 31 | 10 | 10 | 27 | 11 | — |

*(d) The effect of change in acidity due to bacterial action.*

A solution of spray-dried milk was inoculated with *Streptococcus lactis* and incubated at 30°. At different times after the inoculation, samples of the milk were removed, the titratable acidity and the $p_H$ determined, and a volume

Table IV. *The effect of change in acidity due to bacterial action.*

| | $p_H$ | | Acidity calculated as % of lactic acid | Dialysable Ca and P: Calculated on non-fatty solids of original milk | | | Dialysable Ca and P: Calculated on total present in milk | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample | Inoculated milk | Dialysate | | Ca | Inorg. P | Org. P (by diff.) | Ca % | Inorg. P % | Org. P (by diff.) % |
| *a* | 6·55 | 6·55 | 0·14 | 0·46 | 0·38 | 0·07 | 32 | 34 | 7 |
| *b* | 6·05 | 6·15 | 0·24 | 0·57 | 0·43 | 0·11 | 40 | 39 | 10 |
| *c* | 5·75 | 5·95 | 0·29 | 0·70 | 0·50 | 0·09 | 49 | 45 | 8 |

*Original milk solution.*

| | |
|---|---|
| $p_H$ ... ... ... ... ... ... ... | 6·55 |
| Acidity (calculated as lactic acid) ... ... ... | 0·12 % |
| Total Ca (calculated on non-fatty solids) ... ... | 1·44 % |
| Total P (calculated on non-fatty solids) ... ... | 1·12 % |
| Inorg. P (calculated on non-fatty solids) ... ... | 0·74 % |
| Org. P (calculated on non-fatty solids) ... ... | 0·38 % |

dialysed in the standard manner at 0–5°; it had previously been shown that under such conditions no appreciable change in acidity occurs during the period of dialysis. The results of a typical experiment are given in Table IV.

(*e*) *The effect of heat.*

This effect is deduced from the figures given in Tables II and V. The dried milk (Table II) was a commercial sample obtained by drying the pasteurised milk the dialysis figures of which are given.

## Discussion.

*The effect of dilution.*

Reference to Tables II and III shows that dilution of one part of "reconstructed" pasteurised separated milk with two of water causes an increase of 5–8 % in the dialysable inorganic P and in the dialysable Ca and of double that amount in the dialysable Ca in non-dried pasteurised milk. The difference between the effects of dilution on the dried milk on the one hand and on the pasteurised milk on the other, indicates the presence of some heat or dehydration effect which demands further investigation. The point to be emphasised, however, is that the distribution of the constituents studied is affected by the method of dialysis in so far as the ratio of milk to water in the liquid dialysed is concerned.

It is not suggested that dilution of milk with water has necessarily the same effect as the dilution of the dialysable constituents with water which occurs during dialysis, but the results obtained may be regarded as indicative of the dilution effect which takes place particularly when a small volume of milk is dialysed against a large volume of water. At equilibrium, when the conditions of "static" dialysis as described herein are satisfied, the dialysable constituents both in the sac and in the volume of milk are diluted in the ratio 1000 to 1028, whereas, in such experiments as those recorded by Mattick [1929], the dilution ratio would be 1000 to 7000. It can be concluded, therefore, that considerable differences in the dialysable constituents would be obtained by various workers depending upon the ratio of milk to water used in the experiments. Where a small volume of milk is dialysed against a large volume of water, the results recorded will refer to highly diluted dialysable constituents with possible reaction on the potentially dialysable bodies. Where a large volume of milk is dialysed against a small volume of water, the resultant figures will refer to a practically undiluted milk. The extremes are obtainable by "continuous" dialysis and by "static" dialysis. Reference to the previous paper on the phosphorus distribution shows that whereas 40 % of the total P of a freshly separated non-pasteurised milk was dialysable as inorganic P under "static" conditions, 54 % was dialysable when the "continuous" method was employed. Under "static" conditions limited dilution of pasteurised milk (1 part milk: 2 parts water) causes, according to Table II, an increase in dialysable inorganic P from 40 to 47 %.

Although the present object is merely to indicate the factors which may in part be responsible for the great variation in figures recorded by the numerous workers who have been interested in the problem, it may be remarked that such a dilution effect would be anticipated, but has apparently not been previously considered as a factor of importance. In particular the studies of Holt *et al.* [1925] and of Hastings *et al.* [1926] suggest that dilution would have considerable effect on a system which contains various phosphates in a state of supersaturation.

The dialysable organic P shows a marked difference from the inorganic P in that it is not affected by dilution (see Tables II and III). This is in agreement with results reported in the earlier paper where it was shown that at infinite dilution ("continuous" dialysis method) the dialysable organic P agreed with that obtained by minimum dilution ("static" dialysis method).

This effect is what would be expected from phosphorus organically combined and serves as a confirmation that the difference between the total and the inorganic P figures, determined as indicated, is indeed organically bound P.

*Effect of change in acidity.*

The results, Table IV, show an increase of 11 % in inorganic P and of 17 % in Ca in the dialysable fraction during a change in $p_H$ in the milk from 6·55 to 5·75. Even the comparatively small change in $p_H$ from 6·55 to 6·05 causes increases of 5 % and of 8 % in the dialysable inorganic P and in the Ca respectively.

This would be expected from a consideration of the action of lactic acid on the inorganic constituents of milk. Van Slyke and Bosworth [1915] deduced from a study of milk inoculated with *B. lactis acidi* and *B. lactis aerogenes* under somewhat similar conditions, that the whole of the $CaHPO_4$ (which they consider to be a normal inorganic constituent of milk) passes into solution in 13½ hours. The normal bacterial flora of milk does not, however, consist only of lactic acid-producing organisms, but it is obvious that organisms of this type are of first importance, and it follows that acidity during dialysis must be controlled if reliable figures for the dialysable constituents are to be obtained. The time and temperature of dialysis described above have been shown to satisfy the necessary conditions, and to ensure that acidity changes due to the activity of bacteria, *S. lactis* in particular, are reduced to a minimum.

It may be of interest to note that, according to Table IV, there appears to be an increase in the difference between the $p_H$ of the milk and of the dialysate of that milk as the acidity increases.

The titratable acidity and $p_H$ are dependent to some extent on the dilution as is shown in Table VI which gives the results of such determinations on milk powder "solutions" already examined in Tables II and III.

Table V.

| | Fresh milk | | Sterilised milk | |
|---|---|---|---|---|
| | Original milk | Dialysate | Original milk | Dialysate |
| Calculated on non-fatty solids: | | | | |
| Ca | 1·44 | 0·52 | 1·44 | 0·46 |
| Inorganic P | 0·77 | 0·43 | 0·78 | 0·39 |
| Organic P (by diff.) | 0·24 | 0·11 | 0·23 | 0·10 |
| Calculated on total present in milk: | | | | |
| Ca (%) | 100 | 36 | 100 | 32 |
| Inorganic P (%) | 77 | 43 | 77 | 39 |
| Organic P (by diff.) (%) | 23 | 10 | 23 | 10 |

It will be observed (Table VI) that in all three cases dilution has caused an increase in $p_H$ and a corresponding decrease in titratable acidity when due allowance is made for the dilution. It has previously been shown, Tables II, III and IV, that both dilution and development of acidity cause an increase in dialysable Ca and in inorganic P. It appears, therefore, by comparison with Table VI that dilution of milk, which is accompanied by an increase in $p_H$, causes an increase in the dialysable Ca and in inorganic P notwithstanding the

Table VI. *Change in* $p_H$ *and acidity on dilution.*

| | Powder (2) | | Powder (3) | | Powder (1) | |
|---|---|---|---|---|---|---|
| | Undiluted milk | Diluted milk | Undiluted milk | Diluted milk | Undiluted milk | Diluted milk |
| $p_H$ | 6·45 | 6·70 | 6·40 | 6·65 | 6·50 | 6·75 |
| Titratable acidity calculated as lactic acid (%) | 0·13 | 0·035 | 0·15 | — | 0·15 | 0·037 |
| Titratable acidity calculated as lactic acid on undiluted milk (%) | 0·13 | 0·11 | 0·15 | — | 0·15 | 0·11 |

fact that such an effect is also produced by a decrease in $p_H$ (development of acidity).

It can therefore be inferred that were the $p_H$ maintained constant during the dilution, the effect on the dialysable Ca and P would be even greater than that observed.

It is evident that the acidity effect is an important one and should not only be controlled but also defined when considering the dialysable constituents of milk. For such a definition $p_H$ appears to be the more suitable means since titratable acidity is affected by factors which may differ in different milks, *i.e.* their buffer capacity.

*Effect of heat.*

From the time when the original statement was made that heating of milk caused a precipitation of calcium citrate, various workers have studied the effect of heat on milk. Many of the studies have been undertaken from the practical standpoint, for any such changes are of obvious importance in the "processing" of condensed milk. Whilst the original statement, as far as can be found, has not been confirmed in respect of the actual composition of the body precipitated, the reported results, on the whole, indicate that heat causes a decrease in the soluble calcium salt content of the milk.

Table V offers some support for such a conclusion. It is shown that the dialysable constituents of milk suffer a decrease of 4 % in inorganic P and a similar decrease in Ca when the milk is heated to 100°. (In this particular case the milk was placed in a "steamer" for 1 hour.)

It is doubtful whether the differences recorded in Table II between the pasteurised milk and the powder made from it are significant, but it is difficult to ignore the evidence (the effect of dilution of the pasteurised milk and of the "solution" of the dried milk) that some change had occurred. It appears that whilst heat-treatment reduces the amount of dialysable Ca and P of milk, spray-drying affects the physical state of the normally non-dialysable Ca by rendering it less susceptible to dissolution by dilution.

SUMMARY.

1. Evidence has first been given that the milks and dried milks employed in this investigation do represent "average" milk.

2. A combination of the methods described by other authors for the determination of calcium has been chosen as suitable for this work. Details of the method are given. Phosphorus has been determined by a method described in a previous paper.

3. Dilution of milk causes a decrease in acidity, and, as has been shown by "static" dialysis, results in an increase in the proportion of dialysable calcium and of inorganic phosphorus.

4. Increase in acidity by bacterial action materially increases the proportion of dialysable calcium and of inorganic phosphorus.

5. It follows from conclusions 3 and 4 that a decrease in $p_H$ (by bacterial action) and an increase in $p_H$ (by dilution) both result in an increase in dialysable calcium and inorganic phosphorus.

6. The necessity of suitable acidity control and of appreciation of the effect of dilution has been emphasised since they are factors rendering comparison of the results of many workers impossible.

7. Neither change in acidity nor dilution of the milk appears to affect the dialysable organic phosphorus of the milk.

8. Using the "static" method of dialysis and with controlled conditions, the range, calculating the various ingredients on the total present in separated "average" milk, is as follows:

| | |
|---|---|
| Dialysable Ca ... ... ... ... | 25–33 % |
| Dialysable P—total ... ... ... | 43–51 % |
| Dialysable P—inorganic ... ... | 33–41 % |

These include results from fresh, pasteurised and dried separated "average" milks.

9. The effect of dilution on the calcium is much less in the case of spray-dried milk than in that of pasteurised milk. This appears to indicate either a heat or a dehydration effect.

10. The effect of heat is much less defined, but it appears that the conclusion that heat decreases the amount of soluble phosphorus and of soluble calcium is substantiated.

Our thanks are due to Miss D. F. Filmer, responsible for much of the practical work, and to Messrs J. Lyons and Co., Ltd., in whose laboratories this work was conducted, for permission to publish this paper.

REFERENCES.

Allen (1931). *J. Dairy Res.* **3**, 1.
Bell (1925). *J. Biol. Chem.* **64**, 391.
Fiske and Logan (1931). *J. Biol. Chem.* **86**, 671.
Grosser (1913). *Biochem. Z.* **48**, 427.
Hastings, Murray and Sendroy (1926). *J. Biol. Chem.* **71**, 723.
Holt, La Mer and Chown (1925). *J. Biol. Chem.* **64**, 509.
Jordan, Hart and Patten (1906). *Amer. J. Physiol.* **16**, 268.
Kay and Graham (1933). *J. Dairy Res.* **5**, 63.
Kerridge (1926). *J. Sci. Inst.* **3**, 12, 404.
Kuttner and Lichtenstein (1930). *J. Biol. Chem.* **86**, 671.
Lampitt and Bushill (1931). *Analyst*, **56**, 778.
—— —— (1933). *Biochem. J.* **27**, 711.
—— and Hughes (1924). *Analyst*, **49**, 176.
Lenstrup (1926). *J. Biol. Chem.* **70**, 193.
Magee and Harvey (1926). *Biochem. J.* **20**, 873.
Mattick (1929). *J. Agric. Sci.* **19**, 452.
Morton and Best (1933). *J. Soc. Chem. Ind.* **52**, 6 T.
Pietre and Achard (1931). *Compt. Rend. Acad. Sci.* **193**, 1041.
Rona and Michaelis (1909). *Biochem. Z.* **21**, 114.
Rossi (1930). *Boll. Soc. Ital. Biol. Sper.* **5**, 1060.
Van Slyke and Bosworth (1915). *J. Biol. Chem.* **20**, 135.
—— —— (1916). *J. Biol. Chem.* **24**, 199.
Wardlaw (1914). *J. Roy. Soc. N.S.W.* **48**, 252.
Washburn and Shearer (1932). *J. Biol. Chem.* **99**, 35.
Wha (1934). *Biochem. Z.* **144**, 278.

# CLXXVI. THE EFFECT OF ADMINISTRATION OF CALCIFEROL ON THE INCREASED CALCIUM EXCRETION INDUCED BY THYROXINE.

By LEONARD IRVING PUGSLEY and EVELYN ANDERSON.

*From the Department of Biochemistry, McGill University, Montreal.*

(*Received June 26th, 1934.*)

It has been shown by a number of workers who have followed the excretion of calcium by rats during the administration of irradiated ergosterol, that a decrease in the faecal calcium occurs along with an increase in the excretion of calcium in the urine [Watchorn, 1930; Pugsley, 1932; and others]. Taylor and Weld [1932] showed that a decrease in the faecal calcium of dogs which had received desiccated thyroid occurred during ergosterol administration. The efficacy of irradiated ergosterol therapy in correcting the negative calcium balance in cases of clinical hyperthyroidism has not been so conclusive. Tibbets *et al.* [1932] found no effect, while Hansman and Wilson [1934] found an improvement in the calcium balance. Pugsley and Anderson [1934] have shown that a decrease in the faecal calcium can be produced by the administration of irradiated ergosterol to rats which showed a high faecal excretion of calcium as a result of feeding desiccated thyroid. Although the calcium balance of these rats did not become definitely positive as a result of the ergosterol administration owing to the simultaneous increase in the excretion of calcium in the urine, we considered this a matter of dosage. In this communication we are able to show that a definite improvement in the balance can be obtained when the dosage of the two opposing factors is adequately controlled.

## *Methods.*

The diet, feeding of the rats and analytical methods were the same as those previously described [Pugsley and Anderson, 1934]. Schering-Kahlbaum's thyroxine was dissolved in 0·002 *N* NaOH and made up so that 1 ml. contained 0·1 mg. of thyroxine. The calciferol was kindly given by Messrs Ayerst, McKenna and Harrison. It was dissolved in corn oil and diluted so that 1 ml. contained 50,000 international units.

## *Results.*

The effect of intraperitoneal injections of 0·2 mg. of thyroxine (1 ml. of the above solution night and morning) per rat per day upon the calcium excretion, calcium balance, metabolic rate and body weight of the rats is shown in Table I. The values represent the average of four rats. The thyroxine injections were begun on the 10th day and continued throughout the experiment. The calciferol was introduced on the 40th day and discontinued on the 60th day. This was administered by allowing 0·1 ml. to drop from a pipette into the rat's mouth (0·1 ml. = 5000 international units per rat daily). It may be noted that, until the administration of the calciferol, a progressive increase in the calcium excre-

Table I. *The effect of administration of calciferol on the increased calcium excretion induced by thyroxine.*

| Treatment | Day | Urine-Ca mg./day | Faecal Ca mg./day | Ca balance mg./day | Body wt. g. | % increase in $O_2$ consumption |
|---|---|---|---|---|---|---|
| Control period | 2 | 0·01 | 1·92 | +4·32 | 255 | |
| | 4 | 0·01 | 1·60 | +4·74 | 257 | |
| | 6 | 0·01 | 1·52 | +5·12 | 258 | |
| | 8 | 0·01 | 1·55 | +5·24 | 259 | |
| | 10 | 0·01 | 1·63 | +5·16 | 259 | 0 (normal) |
| 0·2 mg. thyroxine daily from the 10th day throughout the experiment | 12 | 0·01 | 1·96 | +4·28 | 254 | |
| | 14 | 0·03 | 2·65 | +2·94 | 250 | |
| | 16 | 0·03 | 3·20 | +3·42 | 246 | |
| | 18 | 0·05 | 3·72 | +3·23 | 243 | +31·8 |
| | 20 | 0·08 | 5·25 | +2·47 | 240 | |
| | 22 | 0·10 | 4·25 | +3·54 | 240 | |
| | 24 | 0·13 | 5·52 | +2·18 | 238 | +48·5 |
| | 26 | 0·13 | 4·37 | +3·30 | 234 | |
| | 28 | 0·12 | 6·44 | +1·26 | 235 | |
| | 30 | 0·12 | 7·62 | +0·06 | 234 | |
| | 32 | 0·16 | 6·87 | +0·77 | 232 | |
| | 34 | 0·16 | 7·37 | −0·25 | 223 | |
| | 36 | 0·28 | 7·37 | −2·31 | 221 | |
| | 38 | 0·14 | 7·50 | −2·08 | 219 | +46·4 |
| | 40 | 0·17 | 8·62 | −2·39 | 215 | |
| 5000 units calciferol daily | 42 | 0·14 | 7·37 | −0·21 | 209 | |
| | 44 | 0·07 | 5·87 | +1·85 | 206 | |
| | 46 | 0·02 | 6·87 | +0·91 | 203 | |
| | 48 | 0·10 | 6·97 | +0·73 | 199 | +46·4 |
| | 50 | 0·80 | 4·50 | +2·50 | 197 | |
| | 52 | 1·16 | 4·25 | +2·39 | 198 | |
| | 54 | 1·10 | 3·00 | +3·70 | 196 | |
| | 56 | 1·05 | 3·15 | +3·60 | 194 | |
| | 58 | 0·94 | 2·90 | +4·00 | 193 | +32·6 |
| | 60 | 0·96 | 3·03 | +3·81 | 193 | |
| Calciferol discontinued | 62 | 0·52 | 3·43 | +3·85 | 192 | |
| | 64 | 0·45 | 6·50 | +0·85 | 191 | |
| | 66 | 0·37 | 9·25 | −1·82 | 193 | +26·6 |
| | 68 | 0·35 | 11·50 | −4·05 | 194 | |

tion in the faeces occurs, and the calcium balance becomes less positive and finally negative. Upon administration of the calciferol the excretion of calcium in the faeces is definitely decreased within 4 days. It is also noted that the excretion of calcium in the urine does not increase significantly until about the 10th day, and the calcium balance is definitely positive from the 4th day of administration of the calciferol. The rats returned to a negative calcium balance shortly after the calciferol was discontinued.

## Discussion.

This work is in agreement with the previous publication of Pugsley and Anderson [1934] in that thyroxine appears to be just as effective as desiccated thyroid in causing an increase in the excretion of calcium in the faeces in rats. It is also a confirmation of the view of Aub *et al.* [1929] that the thyroid hormone has a specific effect upon calcium metabolism. We do not feel, as Hansman and Wilson [1934] have recently suggested, that the effect of hyperthyroidism on calcium metabolism is determined by the degree of hyperparathyroidism, though our experiments do not, of course, disprove the possibility of concomitant hyperparathyroidism in clinical hyperthyroidism.

The effect of calciferol on the increased excretion of calcium in experimental hyperthyroidism points to a specific effect of vitamin D in controlling the retention of calcium. The fact that we were unable to bring the rats into a positive balance in our previous work by the use of irradiated ergosterol we consider to be due to a failure to control the dosage of the two opposing factors.

SUMMARY.

The intraperitoneal injection of 0·2 mg. of thyroxine daily into adult rats leads to a marked increase in the excretion of calcium in the faeces and the establishment of a negative calcium balance. The administration of 5000 international units of calciferol daily by mouth to these rats reduces the faecal excretion of calcium and corrects the negative calcium balance.

Our thanks are due to Prof. J. B. Collip and Dr D. L. Thomson for their assistance and advice.

REFERENCES.

Aub, Bauer, Heath and Ropes (1929). *J. Clin. Invest.* **7**, 97.
Hansman and Wilson (1934). *Med. J. Australia*, **37**, 81.
Pugsley (1932). *J. Physiol.* **76**, 315.
—— and Anderson (1934). *Biochem. J.* **28**, 754.
Taylor and Weld (1932). *Brit. J. Exp. Path.* **12**, 109.
Tibbetts, McLean and Aub (1932). *J. Clin. Invest.* **11**, 1273.
Watchorn (1930). *Biochem. J.* **24**, 631, 1560.

# CLXXVII. A NEW SATURATED SOLID ALCOHOL FROM THE URINE OF PREGNANT MARES[1].

By GEOFFREY ARTHUR DERING HASLEWOOD, GUY FREDERIC MARRIAN and ERIC RUSSELL SMITH.

*From the Department of Physiology and Biochemistry, University College, London and the Department of Biochemistry, University of Toronto, Canada.*

(*Received June 27th, 1934.*)

The recent proof of the close chemical relationship between the oestrus-producing hormones and the sterols and bile acids by Butenandt *et al.* [1933] has led to the widespread belief that these hormones may be produced in the animal body by the degradation of some sterol-like precursor. Pregnandiol, which was shown by Butenandt *et al.* [1931] to have a sterol-like structure, might be considered as a possible intermediate in the transformation of a sterol or bile acid into one of the oestrus-producing hormones. The facts that it is not present in human male urine or in the urine of non-pregnant human females [Marrian, 1929], and that so far it has only been found in human pregnancy urine together with oestrone and oestriol, lend some support to this view. With the object of providing further support for this theory, a careful examination of the neutral ether-soluble fraction of pregnant mares' urine for pregnandiol was undertaken.

The chilling of an acetone solution of the neutral ether-soluble fraction from human pregnancy urine invariably results, in the experience of the authors, in the deposition of crystals of crude pregnandiol. A similar treatment of the neutral fraction of pregnant mares' urine led us to conclude that either pregnandiol was entirely absent or else it was present in amounts too small to be detected in this manner. It had been noticed, however, that in the ether extraction of the neutral material from aqueous alkaline solution, a dark semi-crystalline solid tended to separate out at the ether-water interface. At first hopes were entertained that this might be crude pregnandiol. This material, however, proved to be much more soluble in acetone than pregnandiol. Treatment of the crude solid with charcoal in alcoholic solution, and finally crystallisation from benzene, yielded white crystals which melted with decomposition at about 300° (*cf.* pregnandiol, M.P. 239°). Subsequently it was found that further quantities of this substance could be obtained by chilling a benzene solution of the main neutral fraction.

Superficially, the new compound resembled pregnandiol to some extent. When heated with concentrated sulphuric acid an orange-red colour with a strong green fluorescence was produced. A similar colour is given under the same conditions by pregnandiol, many of the sterols and many of the bile acids. The new compound was more soluble in ethyl alcohol, methyl alcohol and acetone than was pregnandiol, but less soluble in benzene and ether. Iodine

[1] A preliminary account of this work has previously been published [Smith *et al.*, 1933].

value determinations showed that it was fully saturated. These facts, together with the fact of its higher melting-point, suggested the possibility that the new compound might be of the same type as pregnandiol but with a greater oxygen content.

Combustion figures indicated an empirical formula of $(C_7H_{12}O)_n$. Owing to the great insolubility of the compound in molten camphor, it was impossible to determine its molecular weight by Rast's method. Owing to its low solubility in all the usual organic solvents, molecular weight determinations by other methods were also found to be impossible.

Acetylation in the usual manner with acetic anhydride yielded an acetate, the analysis of which showed that all the oxygen in the molecule of the original substance was present as acetylatable hydroxyl groups. The acetate had a much greater solubility in the usual organic solvents and in molten camphor, and therefore it was hoped that it would be possible to obtain reliable figures for its molecular weight. A number of determinations by Rast's method on a specimen of acetate which had been carefully recrystallised, gave values ranging from 377 to 413. These figures are considerably too low for the triacetate of a substance of the formula $C_{21}H_{33}(OH)_3$, but yet are much too high for the diacetate of a substance $C_{14}H_{22}(OH)_2$. The molecular weight would be in close agreement with the diacetate of a substance containing three oxygen atoms. The combustion figures for the acetate did not, however, allow this explanation of the observed facts.

It seemed possible at first that the original alcohol might not be a single substance and that the acetate obtained might be derived from a single component of the mixture. Hydrolysis of the acetate, however, yielded a substance of the same melting-point as the original alcohol, which gave with the latter a mixed melting-point showing no depression.

Since the determined molecular weight of the acetate cannot be reconciled with any of the possible molecular formulae derived from the carbon and hydrogen determination, the former must be considered as an abnormal value. In this connection it might be pointed out that Smith and Young [1927] observed that several pure organic compounds gave abnormal molecular weights by the Rast method.

The molecular dimensions of the new substance are therefore still in doubt. It seems possible that it is a trihydroxy-substance of the formula $C_{21}H_{33}(OH)_3$, perhaps formed by the hydroxylation of pregnandiol, but so far there is no positive evidence in favour of this attractive hypothesis.

So far no single decomposition product of the new compound has been obtained in appreciable amount. Oxidation by chromic anhydride in glacial acetic acid solution under the mildest conditions apparently caused extensive breakdown of the molecule, yielding a mixture of acidic substances from which it was not found possible to isolate a pure compound. It is interesting to contrast the behaviour of pregnandiol with chromic anhydride; the latter is oxidised smoothly and almost quantitatively to a diketone [Butenandt, 1930].

An attempted dehydration by heating to 250–300° at a pressure of about 12 mm. Hg was complicated by the ease with which the substance sublimed under these conditions. Some dehydration had evidently occurred, since from the products there was isolated a small amount of material (M.P. 123°) which had an iodine value of 62. The mother-liquors from the crystallisation of the latter substance yielded on treatment with bromine in ethereal solution small amounts of an insoluble bromide. It was not possible, however, to obtain large enough amounts of these decomposition products for analysis.

Attempts to prepare the parent saturated hydrocarbon by substitution of the hydroxyl groups with halogen and subsequent reduction have not so far been successful.

It seems doubtful if much progress can be made in elucidating the structure of this interesting compound until much larger amounts are available.

## Experimental.

### *Methods of analysis.*

Carbon and hydrogen determinations were carried out by Dr Ing. A. Schoeller (Berlin) and by Dr H. Stantial of the Chemistry Department, the University of Toronto. The authors are deeply indebted to Dr Stantial for her willing co-operation.

Molecular weights were determined by Rast's camphor method. Some difficulty was encountered in obtaining a specimen of camphor suitable for the purpose. A specimen of camphor sold specially for the purpose was found to be far from uniform, samples taken from different parts of the bottle varying by as much as 2° in M.P. This material, after crystallisation from alcohol and finally after grinding to a paste with pure ether and allowing to dry in the air, was found to be sufficiently uniform for the determinations. Numerous determinations on substances of known molecular weight and of a high degree of purity gave values close to the theoretical. It may be noted that pregnandiol acetate gave perfectly normal molecular weight values.

The authors are greatly indebted to Miss Dorothy Skill for carrying out these determinations.

All samples were dried over $P_2O_5$ *in vacuo* at 80° before analysis.

### *Isolation and purification of the new substance from mares' urine.*

The material used in these experiments was a sample of crude extract of the urine of pregnant mares from which the oestrone fraction had previously been separated. It was very kindly placed at our disposal by Dr André Girard of Paris to whom we wish to express our thanks.

The crude extract was heated for 1–2 hours on a boiling water-bath with strong alcoholic potassium hydroxide solution. The mixture was cooled, diluted with about 3 volumes of water and then ether-extracted after the addition of sodium chloride to facilitate the breaking of the troublesome emulsions that formed. The ethereal extract was washed successively with 2*N* sodium hydroxide, 2*N* hydrochloric acid, 2*N* sodium hydroxide and finally twice with water. The gummy residue obtained by the evaporation of this extract to dryness was dissolved in boiling benzene and the solution then placed in the ice-chest. After 1–2 days, a small amount of dark semi-crystalline solid material was deposited. This was filtered off, washed with cold benzene and then dissolved in hot ethyl alcohol and decolorised by boiling with charcoal. The alcoholic filtrate on evaporation to dryness yielded a nearly white crystalline solid. Remaining traces of gummy and pigmented material were removed by washing with cold benzene and by a further treatment with charcoal in alcoholic solution. The final product obtained in this way showed a rather indefinite M.P. of about 295–299° (decomp.) with preliminary darkening and sintering at about 284°. About 5 g. of this material were obtained from 700 g. of the unsaponifiable ether-soluble material of the urine extract.

Purification of this material was effected in the following way. On cooling a solution in methyl alcohol to 0°, a small amount of a white gelatinous material

was precipitated. This was removed by filtration and the filtrate was then concentrated. To this concentrated methyl alcoholic solution was added an excess of benzene, and the mixture was boiled to lower the relative concentration of methyl alcohol until the point of incipient crystallisation was reached. On cooling to 0° and allowing to stand for 24 hours, the substance separated as small white needles.

A sample recrystallised three times in this way melted at 303–304·5° with decomposition and preliminary sintering some degrees lower. The exact melting-point was found to vary considerably over a range of 5° or so according to the rate of heating and according to the tightness with which the specimen was packed into the melting-point tube. It is clear therefore that the exact melting-point is of only limited value as a criterion of purity.

*Properties and empirical constitution.*

The substance is, like pregnandiol, characterised by an extremely low solubility in most of the common organic solvents. In benzene, light petroleum, hexane and ether the solubility is extremely low, even on boiling. The solubility in ethyl alcohol is rather greater and in methyl alcohol greater still. The best solvents so far found are pyridine and dioxan, but in the cold, solutions of greater strength than 1 % cannot be obtained.

The xanthoproteic reaction was found to be entirely negative, indicating that no aromatic ring is present in the molecule.

The iodine value was determined in the following way. 2·15 mg. were partially dissolved in about 2 ml. of chloroform and then allowed to stand in the dark for 20 minutes in contact with 1·80 ml. of the Rosenmund-Kuhnhenn reagent. Solution was complete immediately after adding the reagent. After the addition of water and 10 % potassium iodide solution, the mixture required 1·875 ml. of *N*/10 thiosulphate to titrate the iodine liberated. A blank experiment took 1·880 ml., showing that the substance was fully saturated.

The optical rotation was determined in pyridine solution in a Fischer 0·5 dm. tube, using the mercury green line. It was not found possible to determine the rotation in methyl alcoholic solution owing to the low solubility of the substance in that solvent. The results are shown in Table I.

Table I. *Optical rotation of new compound* (M.P. *303–304·5°*).

| Weight in mg. | Pyridine ml. | Observed rotation | Temp. ° C. | $[\alpha]_{5161}$ |
|---|---|---|---|---|
| 16·35 | 1·98 | −0·181° | 25 | −43·8° |
| 7·00 | 0·83 | −0·187 | 19 | −44·3 |
| 8·9 | 0·90 | −0·219 | 20 | −44·1 |

(Found: C, 74·77, 74·59; H, 10·81, 10·85 %. $(C_7H_{12}O)_n$ requires C, 74·92; H, 10·79 %. $C_{20}H_{34}O_3$ requires C, 74·45; H, 10·63 %. $C_{22}H_{38}O_3$ requires C, 75·35; H, 10·93 %.)

*Preparation of acetate.* 64·4 mg. of the alcohol were heated to 100° for 2 hours with 5 ml. of acetic anhydride and 1 drop of pyridine. After the addition of an excess of water, the insoluble acetate was filtered off, washed thoroughly, dried *in vacuo* over solid calcium chloride and potassium hydroxide and weighed. 88·6 mg. of material of M.P. 164–166° were obtained. The theoretical yield for the complete acetylation of $(C_7H_{11}OH)_n$ would be 88·5 mg. for this amount.

The material was recrystallised twice from methyl alcohol. 45 mg. of M.P. 168° were obtained.

(Found: C, 70·24, 70·28; H, 9·29, 9·16 %. $(C_7H_{11}OCOCH_3)_n$ requires C, 70·08; H, 9·16 %. $C_{20}H_{31}(OCOCH_3)_3$ requires C, 69·60; H, 8·99 %. $C_{22}H_{35}(OCOCH_3)_3$ requires C, 70·51; H, 9·31 %. $C_{21}H_{34}O(OCOCH_3)_2$ requires C, 71·40; H, 9·60 %. $C_{21}H_{35}O_2(OCOCH_3)$ requires C, 72·96; H, 10·13 %. Mol. wt. found: 413, 377, 390, 389, 412, 381; mean 394. $C_{21}H_{33}(OCOCH_3)_3$, $C_{21}H_{34}O(OCOCH_3)_2$ and $C_{21}H_{35}O_2(OCOCH_3)$ require 462, 420 and 378 respectively.)

*Hydrolysis of acetate.* 18·5 mg. of the acetate were boiled with 5 ml. of 10 % alcoholic potassium hydroxide for 3 hours. Excess of water was then added and the precipitated material was filtered off, washed and dried. After recrystallisation from benzene-methyl alcohol the product melted at 298°, and mixed with the original alcohol melted at 301°.

## Summary.

1. From the neutral, ether-soluble fraction of pregnant mares' urine there has been isolated a saturated alcohol, M.P. 303–304·5; $[\alpha]_{5461}$, −44°. Analyses are in good agreement with the formula $(C_7H_{12}O)_n$.

2. On acetylation an acetate, M.P. 168°, was obtained which analysed to the formula $(C_7H_{11}O.COCH_3)_n$. Hydrolysis of the acetate yielded the original alcohol.

3. It has not so far been possible to determine satisfactorily the molecular weight of either the alcohol or its acetate.

4. The possibility of the alcohol being closely related to pregnandiol is discussed.

## REFERENCES.

Butenandt (1930). *Ber. deutsch. chem. Ges.* **63**, 659.
—— Hildebrandt and Brücher (1931). *Ber. deutsch. chem. Ges.* **64**, 2529.
—— Weidlich and Thompson (1933). *Ber. deutsch. chem. Ges.* **66**, 601.
Marrian (1929). *Biochem. J.* **23**, 1090.
Smith and Young (1927). *J. Biol. Chem.* **75**, 289.
—— Hughes, Marrian and Haslewood (1933). *Nature*, **132**, 102.

# CLXXVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

## XXXIX. THE METABOLIC PRODUCTS OF *PENICILLIUM CRATERIFORME* GILMAN AND ABBOTT.

By ALBERT EDWARD OXFORD and HAROLD RAISTRICK.

*From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.*

(*Received June 28th, 1934.*)

Spiculisporic acid ($C_{17}H_{28}O_6$, the $\gamma$-lactone of $\gamma$-hydroxy-$\beta\delta$-dicarboxypentadecanoic acid) was first isolated by Clutterbuck *et al.* [1931] as a metabolic product of *P. spiculisporum* Lehman, when grown on Czapek-Dox solution with glucose as sole source of carbon. They also determined its structural formula. Later, Birkinshaw and Raistrick [1934] showed that this acid together with a related acid, minioluteic acid, were formed when *P. minioluteum* Dierckx was grown on the same medium. Our colleague, Mr G. Smith, recently noticed that when *P. crateriforme* Gilman and Abbott [1927] was grown on Czapek-Dox medium in test-tubes, the metabolism solution soon gave a characteristic reaction in that a heavy precipitate was formed on addition of ferric chloride solution. After following the usual procedure of growing the mould in flasks, we have isolated the metabolic product responsible for this reaction and have found that it is in fact spiculisporic acid. Although *P. crateriforme* is, according to Thom [1930], more closely related morphologically to *P. minioluteum* than to *P. spiculisporum*—all being members of the Biverticillata-Symmetrica division of species of *Penicillium*, but only the first two species being placed in the Funiculose series—yet no trace of minioluteic acid, or of any other similar complex aliphatic acid, could be isolated from its metabolism solution. As regards metabolism of glucose, therefore, *P. spiculisporum* and *P. crateriforme* are very closely related biochemically, the yield of spiculisporic acid being of the same order of magnitude for each species, *viz.* about 0·16 g. per litre of Czapek-Dox medium, but the first-named mould appears to metabolise glucose much faster than the latter. Succinic acid is also a metabolic product of both moulds. *P. crateriforme* synthesises from glucose a complex carbohydrate which is being further investigated.

### Experimental.

A number of 1 litre conical flasks, each containing 350 ml. of Czapek-Dox solution of the following composition: glucose, 50 g.; $NaNO_3$, 2 g.; $KH_2PO_4$, 1 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; water, to 1000 ml., were sterilised and sown with a spore suspension of *P. crateriforme* Gilman and Abbott and incubated at 24°. The organism was purchased from the Centraalbureau voor Schimmelcultures at Baarn, Holland, in November 1933 and had been received by them directly from Abbott.

Growth was slow and in most flasks the initial thin mycelial felt was just complete in 6 days. Thereafter, successive layers of greyish, slimy mycelium developed and in turn fell to the bottom of the flask. In the later stages of metabolism the surface mycelium became faintly pink and in some flasks became brick-red at the edge. One flask was removed after each of the following periods of incubation: 6, 12, 20, 28, 49, 57 and 84 days. The following estimations and tests were carried out on the filtered metabolism solution: $p_H$; titratable acidity; apparent % glucose by polarimeter; % glucose by the Shaffer-Hartmann method; bromine absorption value by Koppeschaar's method; and reactions with the following reagents (mostly in dilute solution) added drop by drop to 2 ml. of the metabolism solution: the sulphates of Cu, Zn, Cd, Ni, Co, Mn$^{\cdot\cdot}$; the chlorides of Mg, Ba, Fe$^{\cdot\cdot\cdot}$, and Hg$^{\cdot\cdot}$, the nitrates of Ag, Li and Sr, and the acetates of U, Ca and Pb (normal and basic); sodium hypochlorite; bromine water; dilute potassium permanganate; Brady's 2 : 4-dinitrophenylhydrazine reagent; dilute HCl; and ethyl alcohol (4 vols.).

The only striking reactions which developed during the course of metabolism were the formation of precipitates with HCl (slight), $FeCl_3$ (especially good greyish brown precipitate), $CuSO_4$ (bluish green, much less copious than the $FeCl_3$ precipitate), and ethyl alcohol (precipitation of a polysaccharide). No reactions were given with the other reagents. The salient results are summarised in Table I, from which it will be seen that glucose is slowly metabolised during some 7 weeks; the $p_H$, after an initial fall, remains constant at about 3·5; the titratable acidity is never very great; the bromine absorption value is negligibly small; no appreciable amount of highly optically active material is to be found in the metabolism solution when all the glucose has been metabolised, although evidence of the formation of a polysaccharide was obtained.

*Isolation of spiculisporic acid from the Ba and Fe precipitates.* After 49 days' incubation, the precipitate given by addition of excess of 2$N$ $FeCl_3$ to some 250 ml. of filtered metabolism solution was collected, washed with water and triturated with 2$N$ $H_2SO_4$, the acid solution being then extracted twice with ether. The oil obtained by evaporation of the dried ether extract became solid when treated with boiling light petroleum (B.P. 40–50°) and was then crystallised from water to yield a small amount of almost colourless, shining plates, melting at 140–143° alone, and at 140–145° when mixed with authentic spiculisporic acid (M.P. 145–146°).

In order to isolate a quantity of the metabolic product sufficient for complete identification, 13 flasks were removed after 62 days' incubation, and to the total filtered metabolism solution was added, first of all, an excess of 6 % barium nitrate solution. The relatively slight precipitate (2·66 g.) which should contain barium minioluteate if the metabolism solution contained miniolueic acid [see Birkinshaw and Raistrick, 1934] appeared to consist chiefly of $BaSO_4$, and was triturated with excess of 2$N$ HCl and then extracted three times with much ether, the undissolved part not being filtered off before extraction. Evaporation of the dried ether extract yielded 0·1 g. of an oil which later partly solidified. After three crystallisations from water, shining plates of almost pure spiculisporic acid separated, M.P. 142–144°; mixed with authentic spiculisporic acid (M.P. 145–146°), the M.P. was 142–146°. No other crystalline product could be isolated by slow evaporation of the various aqueous mother-liquors.

The filtrate from the barium precipitate was treated with excess of ferric nitrate solution and, after standing overnight, the copious greyish brown precipitate was filtered off, washed with water and triturated with 2$N$ $H_2SO_4$, the resulting solution being extracted three times with much ether. The filtrate

Table I.

| Period of incubation Days | $p_H$ | Titratable acidity per 10 ml. ml. $N/10$ NaOH | Apparent glucose by polarimeter % | Glucose by Shaffer-Hartmann % | Bromine absorption value mg. per ml. | Physical properties of metabolism solution | Precipitates with | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | HCl | $FeCl_3$ | $CuSO_4$ | EtOH (4 vols.) |
| 6 | 4·3* | 0·4 | 4·64† | — | 0·09 | Colourless | No precipitate | No precipitate | Slight gelatinous precipitate | Faint turbidity |
| 12 | 3·7 | 1·3 | 4·16 | 4·23 | 0·17 | Do. | Definite flocculent precipitate | Do. | Slight precipitate | Slight turbidity |
| 20 | 3·5 | 1·7 | 3·32 | — | — | Colourless. Definitely more viscous than control | Slight colourless amorphous precipitate | Moderate greyish amorphous precipitate | Moderate bluish green precipitate | Marked turbidity |
| 28 | 3·3 | — | 3·0 | 3·19 | 0·41 | Do. | Do. | Good greyish amorphous precipitate | Do. | Do. |
| 49 | — | — | 1·02 | — | 0·5 | Colourless. Definitely not as viscous as after 28 days | Do. | Do. | Only turbidity | Marked turbidity. Precipitate settles on standing |
| 57 | 3·4 | — | 1·66 | — | — | Do. | Do. | Do. | Do. | Do. |
| 84 | 3·7 | 3·55 | 0·03 | 0·11 | — | Do. | Moderate precipitate | Heavy precipitate | Moderate precipitate | Do. |

* Initially 4·2.

† 5·08 for unsown medium.

from the Fe precipitate gave no further precipitate with lead or copper nitrate solutions. Evaporation of the dried ether extract yielded 2·0 g. of a pasty solid which, after three crystallisations from about 40 ml. $H_2O$, yielded almost colourless slender needles (0·32 g.), M.P. 141–143°. Mixed with spiculisporic acid, the M.P. was 142–146°.

0·3019 g. of this product, dissolved in 20·0 ml. absolute alcohol in a 1 dm. tube gave a rotation of −0·23° with the mercury green light. Hence, $[\alpha]_{5461} - 15°$; Clutterbuck *et al.* [1931] give for spiculisporic acid $[\alpha]_{5461} - 14{\cdot}76°$. The above alcoholic solution was titrated in the cold using phenolphthalein as indicator. 18·05 ml. *N*/10 NaOH were required; equivalent = 167 (theoretical, 164). For final confirmation of the identity of the product with spiculisporic acid, 0·2605 g. of the acid recovered from the above titration (M.P. 139–142°) was dissolved in 15·9 ml. *N*/10 NaOH (*i.e.* just enough for neutralisation) made up to 20 ml., and the rotation of resulting solution observed in a 2 dm. tube. Found: $\alpha + 0{\cdot}48°$, hence $[\alpha]_{5461} + 18°$; Clutterbuck *et al.* [1931] give $[\alpha]_{5461} + 14{\cdot}98°$ for the sodium salt.

No crystalline product other than spiculisporic acid was isolated by slow evaporation of the various aqueous mother-liquors.

*Estimation of yield of spiculisporic acid.* Twenty-nine flasks were removed after 90 days' incubation (residual glucose, nil). No spiculisporic acid could be isolated by extraction of the dried mycelium with boiling water. The filtered metabolism solution was acidified with HCl and the dark, slimy precipitate collected and repeatedly extracted with boiling water until nothing further crystallised from the extract when placed in the cold-room overnight; yield, 1·6 g. of nearly pure spiculisporic acid ≡ 0·16 g. per litre.

*Isolation of succinic acid from the metabolism solution.* The acid filtrate from the precipitate of crude spiculisporic acid was evaporated to small bulk *in vacuo* at 50° and then extracted many times with ether. Evaporation of the dried ether extract yielded a dark-coloured oil which on long standing deposited some crystalline material, which was collected by draining on a porous tile and then taken up in alcohol; the crystalline material left after slow evaporation of the filtered alcoholic solution was sublimed at 110–140° in a high vacuum. The crystalline sublimate melted at 187–189°, alone or mixed with authentic succinic acid (M.P. 188–189°).

## SUMMARY.

The metabolic products of *Penicillium crateriforme* Gilman and Abbott, when grown at 24° on Czapek-Dox solution containing glucose as sole source of carbon, have been investigated. Spiculisporic acid ($C_{17}H_{28}O_6$, the $\gamma$-lactone of $\gamma$-hydroxy-$\beta\delta$-dicarboxypentadecanoic acid), succinic acid and a complex polysaccharide, which is being further investigated, were isolated.

## REFERENCES.

Birkinshaw and Raistrick (1934). *Biochem. J.* **28**, 828.
Clutterbuck, Raistrick and Rintoul (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 301.
Gilman and Abbott (1927). *Iowa State Coll. J. Sci.* **1** (3), 225.
Thom (1930). The Penicillia. (Baillière, Tindall and Cox, London.)

# CLXXIX. NOTE ON THE USE OF DIOXAN AS SOLVENT IN THE DETERMINATION OF MOLECULAR WEIGHTS BY THE CRYOSCOPIC METHOD.

By ALBERT EDWARD OXFORD.

*From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.*

(*Received June 28th, 1934.*)

In the course of work carried out in this laboratory on the constitution of complex mould metabolic products—many of which are polybasic acids, or otherwise highly oxygenated compounds, very sensitive to the action of heat or mild reagents—the need for a neutral, chemically inert, non-hydroxylic liquid of extensive solvent powers and of convenient freezing-point just below room temperature, has often been felt. With such a solvent it would be possible to determine the molecular weight of almost any organic compound quickly and accurately. The need arises because many mould metabolic products are not sufficiently soluble in cold benzene, ethylene dibromide or nitrobenzene to allow of an accurate determination of the molecular weight by the depression of the freezing-points of these solvents, and other solvents such as water and glacial acetic acid are obviously unsuitable for this type of compound. Further, the Rast camphor method cannot be used with compounds which decompose below 170°, and has indeed often yielded abnormal results with polybasic acids apparently quite stable at this temperature. It has been found that the properties of dioxan (diethylene dioxide, $O:(CH_2.CH_2)_2:O$; B.P. 101·4°; F.P. 11·67°; density, 1·0) closely approach those of the ideal solvent mentioned above. This observation is not new, but appears not to be generally known since, as far as the author is aware, it has not previously been explicitly stated.

Dioxan is a colourless, stable liquid, chemically quite inert, the solvent powers of which at room temperature are wide and unique. Its freezing-point is very conveniently just below the ordinary room temperature and its molecular depression constant ($K$) is relatively large. Furthermore, no compound (neutral, acidic or basic) so far tested dissolves in dioxan to give a solution containing any considerable proportion of polymerised or dissociated molecules, provided that the solute contains no water or other solvent of crystallisation. Since dioxan is only slightly hygroscopic, it follows that reliable molecular weights can be determined in the usual Beckmann apparatus.

The use of dioxan for this purpose was first mentioned by Anschütz and Broeker [1926] and the method for its purification was improved by Eigenberger [1931], but these authors appear not to have made a detailed study of its solvent powers. Briefly it may be stated that hydrocarbons, sterols, alcohols and carbonyl compounds of the aliphatic and terpene series, aliphatic and aromatic mono- and poly-basic and hydroxy-acids (free or esterified), aromatic nitro-compounds, phenols (free, methylated or acetylated), amides (*e.g.* hippuric acid)

and many bases are in general so readily soluble in cold dioxan that depressions in F.P. of 0·4° or more can be obtained. Some of the results on which this statement is based are collected in Table I, from which it can be seen that the

Table I.

$K = \frac{\Delta M}{C}$, where $M$ = molecular weight of solute.
$\Delta$ = depression in F.P. in ° C.
$C$ = g. solute dissolved in 100 g. dioxan.

| Solute | $M$ | $C$ | $\Delta$ | $K$ |
|---|---|---|---|---|
| Neutral substances: | | | | |
| 1. Choral hydrate. $CCl_3.CH(OH)_2$ | 165·4 | 3·264 | 0·936 | 47·4 |
| 2. Triphenylmethane. $C_{19}H_{16}$. M.P. 92° | 244·1 | 3·885 | 0·754 | 47·4 |
| Phenols: | | | | |
| 3. 6 : 8-Dihydroxy-3-methyl*iso*coumarin. $C_{10}H_8O_4$ | 192 | 0·998 | 0·235 | 45·2 |
| 4. Quinol. $C_6H_6O_2$ | 110 | 1·891 | 0·813 | 47·3 |
| 5. Kojic acid. $C_6H_6O_4$ | 142 | 2·079 | 0·663 | 45·3 |
| Acids: | | | | |
| 6. Benzoic acid. $C_7H_6O_2$ | 122 | 1·780 | 0·678 | 46·5 |
| 7. Byssochlamic acid. $C_{18}H_{20}O_6$ [Raistrick and Smith, 1933] | 332·2 | 3·493 | 0·472 | 44·9 |
| 8. Carlic acid. $C_{10}H_{10}O_6$ [Clutterbuck *et al.*, 1934] | 226 | 1·814 | 0·352 | 43·6 |
| 9. Fumaric acid. $C_4H_4O_4$ | 116 | 1·398 | 0·578 | 48·0 |
| 10. 6-Methylsalicylic acid. $C_6H_3(OH)(CH_3)(COOH)$ | 152 | 1·956 | 0·595 | 46·2 |
| 11. Mycophenolic acid. $C_{17}H_{20}O_6$ | 320·2 | 3·641 | 0·521 | 45·8 |
| 12. Palmitic acid. $C_{15}H_{31}.COOH$ | 256·2 | 3·840 | 0·722 | 48·1 |
| 13. Spiculisporic acid. $C_{17}H_{28}O_6$ | 328·2 | 5·799 | 0·857 | 48·5 |
| 14. Succinic acid. $C_4H_6O_4$ | 118 | 0·954 | 0·377 | 46·6 |
| 15. Terreic acid. $C_8H_{10}O_5$ [Raistrick and Smith, 1934] | 154 | 2·702 | 0·815 | 46·5 |
| 16. Tartaric acid. $C_4H_6O_6$ | 150 | 4·344 | 1·337 | 46·2 |
| Base: | | | | |
| 17. Caffeine (anhydrous). $C_8H_{10}O_2N_4$ | 194 | 1·184 | 0·297 | 48·7 |
| | | | Mean | 47 |

value of the constant $K$ is, with a few exceptions to be discussed later, always very close to 47, the mean of the most reliable determinations. This value also agrees well with that obtained by Eigenberger (*viz.* 48) who tested only four solutes. The following types of compounds are however not sufficiently soluble in cold dioxan (*i.e.* they give a saturated solution of concentration less than 0·04 $M$ whose F.P. is less than 0·2° below that of pure dioxan) for an accurate determination of their molecular weights to be made: amino-acids (*e.g.* leucine, aspartic acid); alkali salts of organic acids; carbohydrates (*e.g.* glucose, arabinose); glucosides (*e.g.* salicin); sugar alcohols (*e.g.* mannitol, sorbitol); anthraquinone; certain mould pigments related to anthraquinone (*e.g.* helminthosporin) or xanthone (*e.g.* citromycetin); urea; certain complex bases (*e.g.* cinchonine); and salts of bases (*e.g.* aniline hydrochloride, quinine sulphate). All these compounds, with the exception of the amino-acids, alkali salts and carbohydrates, are however appreciably (some readily) soluble in boiling dioxan and crystallise well on cooling, so that their molecular weights could doubtless have been determined by the ebullioscopic method with dioxan as solvent (for which $K = 22$ according to Anschütz and Broeker), provided that the solute is stable at 101°.

It will be noticed that certain solutes in Table II, *viz.* citric and oxalic acids, the hydrated form of 3 : 5-dihydroxy-2-carboxybenzoylmethylketone [Oxford and Raistrick, 1933] and brucine give abnormally high values for $K$. These all contain water of crystallisation and the affinity of dioxan for water is such that water of crystallisation is split off from the solute molecules. Since the

constant is in no case as high as it ought to be if complete dissociation had taken place, it follows that the water molecules are shared between solvent and solute. It is worthy of note that with chloral hydrate as solute a perfectly normal value for $K$ is obtained. The accepted idea that that compound contains water of constitution and not of crystallisation is thus confirmed. On the other hand, the molecular compound $CH(C_6H_5)_3$, $C_6H_6$ (triphenylmethane crystallised from benzene) is completely dissociated in dioxan solution, the value for $K$ being almost exactly twice that for normal compounds.

The only solute so far tested which appears to polymerise appreciably in dioxan solution (or to form mixed crystals with the solvent) is carolic acid (see Table II). The abnormality in this case is however not very great, since, using the mean value of $K=47$, the molecular weight of carolic acid is found to be 209 instead of 182.

Table II. *Substances which give abnormal values for K when dissolved in dioxan.*

| | Solute | $M$ | $C$ | $\Delta$ | $K$ |
|---|---|---|---|---|---|
| Associated: | | | | | |
| | Carolic acid. $C_9H_{10}O_4$ [Clutterbuck *et al.*, 1934] | 182 | 3·978 | 0·894 | 40·9 |
| Dissociated: | | | | | |
| 1. | Triphenylmethane (crystallised from $C_6H_6$). $C_{19}H_{16}$, $C_6H_6$. M.P. 76° | 322·2 | 3·459 | 0·993 | 92·5 |
| 2. | Citric acid. $C_6H_8O_7$, $H_2O$ | 210 | 2·293 | 0·911 | 83·4 |
| 3. | Hydrated form of 3 : 5-dihydroxy-2-carboxybenzoyl methyl ketone. $C_{10}H_8O_6$, $H_2O$ | 242 | 4·107 | 1·363 | 80·3 |
| 4. | Minioluteic acid. $C_{16}H_{26}O_7$. Air-dried. (Contains some $H_2O$ [Birkinshaw and Raistrick, 1934]) | 330·2 | 3·329 | 0·818 | 81·1* |
| 5. | Oxalic acid. $C_2H_2O_4$, $2H_2O$ | 126 | 1·481 | 1·377 | 117·2 |
| 6. | Brucine. $C_{23}H_{26}O_4N_2$, $4H_2O$ | 466·3 | 1·657 | 0·372 | 104·7 |

* Minioluteic acid after drying at 110° for 3½ hours gave an almost normal value for $K$, *viz.* 53·5.

Since dioxan may fairly be considered to supersede all the solvents usually employed for the determination of molecular weights by the cryoscopic method, it appeared feasible to develop a semi-micro method by replacing the customary Beckmann wide-bulbed thermometer by an ordinary thermometer with a narrow bulb (size of bulb 5 × 0·7 cm.), graduated in 0·02° over the range 10–12° only. Freezing-points were determined in a small test-tube (15 × 1¼ cm.) fitted with a cork carrying the thermometer, and for stirrer a length of nichrome wire (S.W.G. 26, such as is used for making platinum loops for bacteriological work) suitably bent at the lower end, was used. Its upper end was pushed into a cork in order that heat from the hand should not be conducted into the liquid, and a small groove was cut in the thermometer cork, through which the wire passed. The volume of solvent required was 5 ml. and reliable results were obtained with this simple apparatus by observing the following points:

(*a*) Stirring should be slow throughout, otherwise too much heat is developed by friction.

(*b*) No attempt should be made to cause the liquid to freeze until it is supercooled at least 0·4°.

The great advantage of this apparatus is that when freezing does take place the mercury thread rises very swiftly to a maximum at which it remains for a considerable time. The results obtained are given in Table III. No doubt a satisfactory micro-method could be devised by using a micro-Beckmann

Table III. *Molecular weights determined by the semi-micro method.*

| Substance | Weight of substance in mg. | Weight of dioxan in g. | Δ | *M* (obs.) | *M* (theor.) |
|---|---|---|---|---|---|
| Benzoic acid | 40·4 | 5·61 | 0·292 | 116 | 122 |
| *p*-Nitraniline | 43·8 | 5·25 | 0·287 | 137 | 138 |
| Flavoglaucin. $C_{19}H_{28}O_5$ [Gould and Raistrick, 1934] | 90·2 | 5·51 | 0·267 | 288 | 304 |
| Auroglaucin. $C_{19}H_{32}O_5$ [Gould and Raistrick, 1934] | 88·5 | 5·36 | 0·263 | 295 | 298 |
| Citrinin. $C_{13}H_{14}O_5$ | 68·5 | 5·53 | 0·225 | 259 | 250 |

thermometer and an apparatus suitably designed for protection of the solvent from atmospheric moisture.

Since dioxan is miscible in all proportions with both water and light petroleum, most solutes may be recovered quantitatively by addition of one or other of these solvents.

## Experimental.

### *Purification of dioxan.*

Technical dioxan (B.D.H.), which contains considerable amounts of acetaldehyde and ethylene acetal, was purified by the method given by Eigenberger [1931]. 900 g. were boiled with *N* HCl (90 g.) during two working days, a slow stream of air being continuously drawn through the condenser to remove acetaldehyde formed by hydrolysis of the acetal. At the end of this period, a test portion of the liquid still gave a positive reaction with Brady's 2: 4-dinitrophenylhydrazine reagent, although not nearly as strongly as at the beginning. Water and HCl were removed by treatment with solid KOH, the dioxan top layer being separated and further dried over solid KOH and finally over sodium for a day. The product was finally distilled over sodium, the distillate being collected in receivers protected from atmospheric moisture by $CaCl_2$-tubes. The first fraction (B.P. 101–101·2°, 57 g.) gave a positive reaction with Brady's reagent and was not used for molecular weight determinations. The next fraction (B.P. 101·2–101·4°; 168 g.) had F.P. 11·5° and gave no reaction with Brady's reagent. The third and main fraction (525 g.) boiled constantly at 101·4° and had F.P. 11·67°* and moreover behaved like a pure liquid at its F.P. It gave no reaction with Brady's reagent. It was also noticed that just before the changing of the receivers previous to the collection of the main fraction, the sodium in the distillation flask had assumed the form of numerous silver-bright, smooth spheres. The end fraction (B.P. 101·4°) weighed only 31 g. The main fraction was regarded as pure dioxan and was used for the determination of most of the values of *K* recorded in Table I.

### *Method of determination of* F.P.

Since dioxan is appreciably hygroscopic, the following modified method was used. A large quantity of the pure solvent was prepared and stored in a bottle with a well-fitting stopper. The freezing-point on the scale of a Beckmann thermometer appropriately set was determined once and for all and noted on the label, and was found not to vary more than a few thousandths of a degree over several months. The solute is finely powdered and weighed directly into a boiling-tube, which is afterwards corked and weighed. The requisite volume of

* According to Eigenberger, pure dioxan melts at 12·5–13°.

solvent is then added and the tube immediately corked and re-weighed; then gently shaken until all the solute has dissolved, with gentle warming if necessary. The cork is then replaced by another bearing the thermometer and stirrer (a nickel wire) and the F.P. determined immediately. The first reading is generally a few thousandths of a degree higher than the next and so on, since slow absorption of atmospheric moisture takes place, even if the glass tube through which the stirrer passes is fitted with narrow rubber tubing to encase the upper part of the stirrer. The highest reading (almost always the first) is recorded as the F.P. of the solution. The setting of the Beckmann thermometer must of course not be altered during the whole time this bottle of solvent is in use.

SUMMARY.

Many types of crystalline organic compounds, including most natural products other than sugars and amino-acids, are so readily soluble in cold dioxan that this solvent, being chemically quite inert, may not only supplement the use of, but also advantageously replace, all the others usually employed for the determination of molecular weight by the cryoscopic method. Pure dioxan has F.P. 11·67° and its molecular depression constant, $K$, is equal to 47.

The author thanks Prof. H. Raistrick for much helpful encouragement.

REFERENCES.

Anschütz and Broeker (1926). *Ber. deutsch. chem. Ges.* **59**, 2844.
Birkinshaw and Raistrick (1934). *Biochem. J.* **28**, 828.
Clutterbuck, Haworth, Raistrick, Smith and Stacey (1934). *Biochem. J.* **28**, 94.
Eigenberger (1931). *J. prakt. Chem.* **130**, 78.
Gould and Raistrick (1934). *Chem. Ind.* **53**, 277.
Oxford and Raistrick (1933). *Biochem. J.* **27**, 634.
Raistrick and Smith (1933). *Biochem. J.* **27**, 1814.
—— —— (1934). *Chem. Ind.* **53**, 451.

# CLXXX. PRELIMINARY NOTE ON THE ACTION OF GASES UNDER PRESSURE ON THE GROWTH OF MICRO-ORGANISMS.

## I. ACTION OF OXYGEN UNDER PRESSURE AT VARIOUS TEMPERATURES.

By AAGE CHRISTIAN THAYSEN.

*From the Microbiological Section, Chemical Research Laboratory, Teddington.*

(*Received June 11th, 1934.*)

The first observations on the action of oxygen at high pressure on the respiratory functions of animals and plants were made by the French physiologist Bert [1872; 1873, 1, 2]. He found that the consumption of oxygen by animals placed in oxygen under 3·5 atmospheres' pressure, or in air under correspondingly higher pressures, fell far short of the normal and that such animals succumbed rapidly with symptoms resembling those of strychnine poisoning.

The subjects of putrefaction and of fermentation were foremost in the minds of many biologists at that time, and Bert extended his researches from the behaviour of higher animals and plants to that of the action of high pressure oxygen on putrefaction.

In a final paper Bert [1875] reported that he had succeeded in keeping a piece of raw meat free from putrefaction for a prolonged period by storing it in a container under an oxygen pressure of 15 atmospheres. Raw eggs were similarly preserved. In the case of the meat the oxygen affected the colour, turning the normal red into a yellowish amber, while the taste of the meat became somewhat flat.

Bert [1875] also attempted to ascertain what effect, if any, oxygen under pressure would have on various enzymes. He concluded from his observations that vegetable diastase, pepsin and the enzymes of saliva and of the pancreatic juice remain unaffected under these conditions. Further, he pointed out that the protective action of oxygen and of air under high pressures was a function of the abnormally increased concentrations of oxygen which entered the living tissues under these conditions and not of the pressures as such. In other words he believed the action to be one typical for oxygen and not necessarily inherent in other gases.

Subsequently Nourry and Michel [1892] reported that milk, kept in the cold and under a high pressure of carbon dioxide, would remain sweet for as long as 8 days owing to a retarding action of the carbon dioxide on the growth of the microflora of the milk.

An inhibitory action of carbon dioxide on micro-organisms was observed also by Hoffmann [1906] who claimed that *Bact. coli commune*, *Bact. typhosum* and *Vibrio cholerae* could be destroyed when subjected to carbon dioxide pressures of from 20 to 50 atmospheres. Hoffmann's observations were followed up by Berghaus [1907] who concluded that carbon dioxide as such, even in the

absence of high pressures, has an inhibitory action on many micro-organisms, an action which increases with the increased quantities of the gas brought into solution on the application of high pressures.

Curiously enough Berghaus reported that the action of carbon dioxide under pressure in his experiments was very much more marked than that of oxygen similarly applied. Organisms like *Bact. coli commune* and *Staphylococcus pyogenes aureus* showed retarded growth when incubated at 37° under an oxygen pressure of up to 75 atmospheres while 15 atmospheres and 9 atmospheres respectively of carbon dioxide applied for 24 hours at 37° were found sufficient to destroy these micro-organisms.

Incomplete and inconclusive as were the above observations they formed the basis for various attempts to apply gases under high pressures for purposes of sterilisation.

The first attempt after that of Bert to apply high pressure gases for purposes of sterilisation was made by d'Arsonval [1891] who claimed to be able to sterilise liquids by passing them through porcelain candles, using high pressure carbon dioxide gas to facilitate the filtration and to weaken the micro-organisms present in the liquids. The weakening of the micro-organisms, he found, was particularly marked when the filtering liquid was kept at increased temperatures (40°).

Schaffer and von Freudenreich [1892] were unable to confirm d'Arsonval's conclusions and report that they failed to sterilise milk by exposure to high pressures of oxygen and carbon dioxide, even when raising the temperature of the milk considerably above 40°. They asserted that even *Bact. typhosum* and vegetative cells of *B. anthracis* remained viable when exposed in milk under such conditions. It should be pointed out that Schaffer and von Freudenreich carried out their experiments under insufficiently controlled conditions, and that their conclusions therefore appear unsupported experimentally.

De Lavallée [1898] obtained more satisfactory results and claimed that he had succeeded in preserving milk for a considerable time and in freeing it from pathogenic micro-organisms by subjecting it first to an exposure for 4–5 hours to a carbon dioxide pressure of from 5 to 6 atmospheres and then to an oxygen pressure of 5 atmospheres for 5 hours. In his experiments the milk was apparently kept cool, since it was taken direct from the cow into cooled containers and then treated. De Lavallée's results were repeated on various occasions but could not be corroborated and Seiffert [1904], Gerasbeck [1905] and Riegel [1905] reported unfavourably on the use of high pressure gases for the sterilisation of milk. Gradually interest in the subject slackened, and little was heard of the bactericidal properties of high pressure gases until Cleveland's [1925] publications on the intestinal fauna of termites, though papers appeared from time to time which testified to the growth-inhibiting action of oxygen on certain types of micro-organisms. Of special interest in this respect are Moore and Williams's [1911] observations that *Mycobacterium tuberculosis* is killed when kept in an atmosphere containing 80 % of oxygen, in conditions in which *Staphylococcus pyogenes aureus* is merely retarded in its development.

Cleveland [1925] succeeded in eliminating the intestinal protozoa of termites by exposing the insects to oxygen pressures of 3·5 atmospheres for periods insufficient to destroy the host itself. He obtained similar results when using air under correspondingly higher pressures, but failed to destroy the protozoa completely when using an air pressure of only 3·5 atmospheres, thus showing that the destructive action was associated with the oxygen and not with the pressure as such. In this respect he confirmed earlier observations, particularly

those of Rogers [1894] who found that pressures of 1000 atmospheres and more may be applied to bacterial cultures without detrimental effect.

In a brief note published subsequently, Cleveland [1926] drew attention to the possibility of utilising the destructive properties of oxygen under pressure for various microbiological purposes such as the sterilisation of culture media. In other words Cleveland reopened the problem of the bactericidal action of high pressure gases in the form in which it had been left by Bert and without, apparently, being aware of the fact that a considerable amount of work had already been done which tended to prove that the destructive properties of high pressure gases are insufficient to justify their practical application. On the other hand it must be admitted that the adverse criticism published prior to Cleveland's investigations was of a very desultory nature.

It is particularly noteworthy to observe when glancing through the earlier observations on the bactericidal action of high pressure gases, that no systematic attempt was ever made to ascertain the relationship of the action of the gas to the various factors which affect the development of all living cells, notably time and temperature, and it was felt that if, as suggested by Cleveland, the question of the use of high pressure gases should be reopened, one of the first investigations to be undertaken would have to be an analysis of the extent to which the duration of the exposure to the gas and the temperature at which the exposure is carried out would affect the bactericidal properties of high pressure gases.

## Experimental.

In the curves shown below certain results have been tabulated, taken from a number of preliminary experiments which were carried out to ascertain the effect of temperature on the action of high pressure oxygen. In these experiments four different types of micro-organisms were used, ranging from a typical aerobic *Bacillus* to a semi-anaerobic *Streptococcus*. For comparison, curves have been plotted also of the growth of the same organisms under identical conditions but left at the test temperatures under ordinary atmospheric conditions.

The curves record the percentage increase and decrease respectively in the numbers of cells present in the cultures in question after 5 hours' incubation at the temperatures recorded on the abscissae, either at ordinary atmospheric pressure or under an oxygen pressure of 10 atmospheres.

It will be observed that in all four cases the high pressure oxygen has had a retarding influence on the growth of the test organisms. But the most noteworthy feature of the curves is that, while at temperatures well below the optimum temperature for growth, the action of the oxygen has been unable to prevent an actual, and sometimes an appreciable, increase in the initial number of cells, a marked decrease has set in where the optimum temperature for growth at atmospheric pressure has been reached or slightly exceeded.

Thus in the case of the vegetative cells of *Bac. mesentericus* (Fig. 1) the raising of the temperature from 24° to 30° has changed a slight but distinct increase in the initial number of cells to a complete destruction of all but the spores present. Of the spores only a small percentage was found to be destroyed by the high pressure oxygen.

In the case of *Bact. coli commune* (Fig. 2) kept under 10 atmospheres' oxygen pressure, it took an increase in temperature of 13°, from 34° to 47°, to change a marked increase in numbers to complete destruction. But this complete destruction was nevertheless achieved at a temperature when the corresponding control culture, kept at atmospheric pressure, still showed an increase in the initial number of cells of nearly 100 %.

Similar, though perhaps somewhat less marked, results were obtained with the two other types of organisms used, *Streptococcus lactis acidi* (Fig. 3), and *Staphylococcus pyogenes aureus* (Fig. 4). In all the four cases it was possible to secure complete destruction within 5 hours of the vegetative cells originally

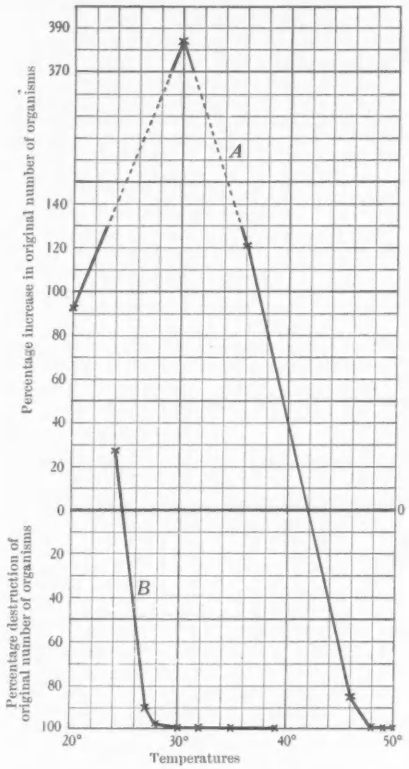



Fig. 1.

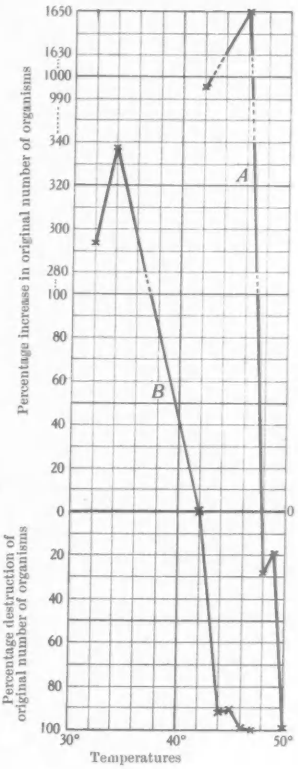



Fig. 2.

Fig. 1. *Bac. mesentericus* (vegetative cells) suspended in ordinary broth ($p_H$ 7·1).

Curve *A*. Percentage increase (decrease) of the original number of cells after 5 hours' exposure to air (atmospheric pressure) at temperatures shown.

Curve *B*. Percentage increase (decrease) of the original number of cells after 5 hours' exposure to oxygen (10 atmospheres' pressure) at temperatures shown.

Fig. 2. *Bact. coli commune*; suspended in 1 % glucose broth ($p_H$ 7·1).

Curve *A*. Percentage increase (decrease) of the original number of cells after 5 hours' exposure to air (atmospheric pressure) at temperatures shown.

Curve *B*. Percentage increase (decrease) of the original number of cells after 5 hours' exposure to oxygen (10 atmospheres' pressure) at temperatures shown.

introduced into the culture at a temperature of 47°, a temperature which was insufficient to eliminate all the initial cells in the controls kept at atmospheric pressures and which should leave coagulable protein and presumably other thermolabile substances unimpaired.

The curves given illustrate why such divergent results as those referred to above should have been obtained by earlier investigators, who, in the majority of cases, were satisfied with testing the action of high pressure oxygen at lower

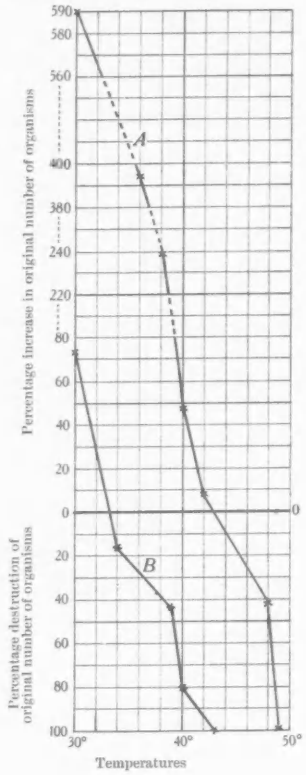



Fig. 3.

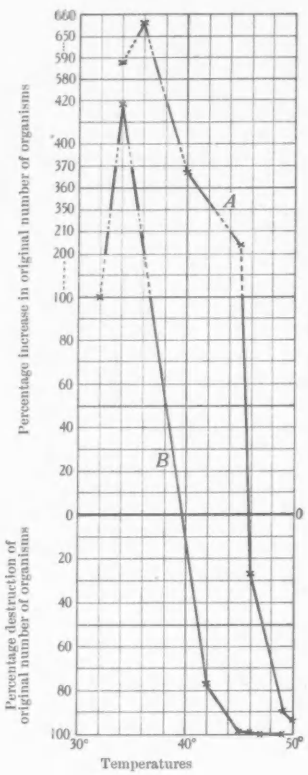



Fig. 4.

Fig. 3. *Streptococcus lactis acidi*; suspended in 1 % glucose broth ($p_H$ 7·1).

Curve *A*. Percentage increase (decrease) of the original number of cells after 5 hours' exposure to air (atmospheric pressure) at temperatures shown.

Curve *B*. Percentage increase (decrease) of the original number of cells after 5 hours' exposure to oxygen (10 atmospheres' pressure) at temperatures shown.

Fig. 4. *Staphylococcus pyogenes aureus*; suspended in broth ($p_H$ 7·1).

Curve *A*. Percentage increase (decrease) of the original number of cells after 5 hours' exposure to air (atmospheric pressure) at temperatures shown.

Curve *B*. Percentage increase (decrease) of the original number of cells after 5 hours' exposure to oxygen (10 atmospheres' pressure) at temperatures shown.

temperatures, *i.e.* at room temperature or below. Why Schaffer and von Freudenreich [1892], who used a temperature as high as 68°, should have failed in their attempts to destroy the non-spore-forming bacteria of samples of milk which they subjected to oxygen pressures is not explained by the curves. But other

experiments carried out by the writer on the influence of the time factor on the bactericidal action of high pressure oxygen appear to indicate that Schaffer and von Freudenreich's negative results were due to the fact that they failed to maintain the increased temperatures for a sufficient time to secure complete destruction. However, before expressing a definite opinion on this point, it will be necessary to carry out a closer study of the bearing of the time factor on the bactericidal action of high pressure oxygen, a study which has not yet been completed.

The present preliminary note was compiled for the purpose of showing that it is possible to confirm experimentally the early observations of Bert and others who attributed bactericidal properties to oxygen at high pressure, and of drawing attention to the need for a careful and complete investigation of the whole of this question with a view, as suggested by Cleveland [1926], to elaborating methods for the destruction of micro-organisms in all such cases where the application of heat or antiseptics would affect the composition of substances such as proteins, enzymes, vitamins and hormones.

SUMMARY.

It has been shown that the following test organisms, *Bac. mesentericus*, *Bact. coli commune*, *Streptococcus lactis acidi* and *Staphylococcus pyogenes aureus*, are very greatly retarded in their growth when this is made to take place in an oxygen atmosphere of 10 atmospheres' pressure at or below the optimum temperature for growth.

On increasing the temperature for growth slightly, by a few degrees, and in some cases as much as 13°, above the optimum temperature for growth the effect of oxygen under pressure on the test organisms was found to be lethal, the gas completely destroying all vegetative cells present in 5 hours or less.

The spores of the strain of *Bac. mesentericus* used were not killed under these conditions.

The writer wishes to express his thanks to the Department of Scientific and Industrial Research and to the Admiralty for permission to publish the above observations.

REFERENCES.

Berghaus (1907). *Arch. Hyg.* **62**, 172.
Bert (1872). *Compt. Rend. Acad. Sci.* **74**, 617.
—— (1873, 1). *Compt. Rend. Acad. Sci.* **76**, 443.
—— (1873, 2). *Compt. Rend. Acad. Sci.* **77**, 531.
—— (1875). *Compt. Rend. Acad. Sci.* **80**, 1579.
Cleveland (1925). *Biol. Bull.* **48**, 455.
—— (1926). *Science*, **63**, 168.
d'Arsonval (1891). *Compt. Rend. Acad. Sci.* **122**, 667.
de Lavallée (1898). *Milchzeitung*, **27**, 472.
Gerasbeck (1905). *Mölkereizeitung*, Berlin, **15**, 184.
Hoffmann (1906). *Arch. Hyg.* **57**, 379.
Moore and Williams (1911). *Biochem. J.* **5**, 181.
Nourry and Michel (1892). *Compt. Rend. Acad. Sci.* **125**, 959.
Riegel (1905). *Mölkereizeitung*, Hilderheim, **19**, 764.
Rogers (1894). *Compt. Rend. Acad. Sci.* **119**, 963.
Schaffer and von Freudenreich (1892). *Centralb. Bakt. Par.* **11**, 346.
Seiffert (1904). *Verhandl. Ges. Kinderheilk.* **21**, 175.

# CLXXXI. THE HEAT-COAGULATION OF CASEINOGEN.

## I. THE RÔLE OF PHOSPHORUS CLEAVAGE.

By GEORGE ROBERTSON HOWAT
and NORMAN CHARLES WRIGHT.

*From the Hannah Dairy Research Institute, Kirkhill, Ayr.*

(*Received June 14th, 1934.*)

Investigations into the heat-coagulation of proteins, notably of egg-albumin and oxyhaemoglobin, indicate that two separate reactions are involved, denaturation and flocculation. Denaturation, which precedes flocculation, involves an irreversible change in the structure of the molecule, probably of a hydrolytic nature. Flocculation is a purely physical phenomenon, being largely dependent on the presence of electrolytes and, under suitable conditions, reversible. Chick and Martin [1912] and Lewis [1926], the former studying egg-albumin and the latter oxyhaemoglobin, have shown that the denaturation of these proteins, as measured by the amount of coagulation in the presence of small quantities of electrolytes, is a unimolecular reaction whose constant $k$ can be accurately determined provided that the $p_H$ of the solution is kept constant throughout the course of the reaction.

Caseinogen is usually regarded as a relatively heat-stable protein, but it is occasionally found that drastic heat treatment (such as occurs in the sterilisation of evaporated milk) will lead to its complete coagulation. Investigations into the causes of such coagulation have in the past been chiefly concerned with the influence of contributory factors, such as the concentration of electrolytes and the presence of protective colloids (*e.g.* lactalbumin) on the heat-stability of the caseinogen. No attempt appears to have been made to study the effect of drastic heat treatment on the protein itself. Yet it is apparent from the results of a number of investigations that the caseinogen molecule is easily disrupted by the action of various agents; more especially is this true of the cleavage of phosphorus from the caseinogen molecule. Thus Rimington and Kay [1926] and Stirling and Wishart [1932] have shown that trypsin-kinase liberates the phosphorus from caseinogen with comparative ease; in two experiments carried out by the latter authors liberation of the entire phosphorus occurred in less than one hour. Rimington and Kay [1926] have also shown that mild alkaline hydrolysis at 37° results in complete dephosphorisation of caseinogen.

It was not therefore surprising to find that, in preliminary experiments in which neutral solutions of sodium caseinogenate were heated in closed tubes at 120°, there was a definite cleavage of phosphorus. It was felt that these preliminary experiments warranted more detailed study. The results of this study are described in the present paper.

## I. The effect of heat on the cleavage of phosphorus from caseinogen.

### *Technique.*

*Preparation of solutions.* Caseinogen "nach Hammarsten" was used throughout. It contained 9 % moisture, the nitrogen and phosphorus contents (calculated on dry matter) being 15·08 and 0·83 % respectively. Solutions were prepared as follows:

*3·5 % sodium caseinogenate.* 3·5 g. caseinogen were mixed with sufficient water and 0·1 *N* NaOH to give, when diluted to 100 ml., a final concentration of 0·0175 *N* NaOH.

*3·5 % calcium caseinogenate.* 3·5 g. caseinogen were mixed with sufficient water and solid $Ca(OH)_2$ to give, when diluted to 100 ml., a final concentration of 0·0175 *N* $Ca(OH)_2$.

The solutions were mechanically stirred until the caseinogen was completely dissolved. The final solutions appeared homogeneous, the solutions of sodium caseinogenate being almost clear and those of calcium caseinogenate opalescent but transparent in thin layers. The $p_H$ of the solutions lay between 6·5 and 7·0. It may be noted here that, immediately they were immersed in the glycerol-bath at 120°, both solutions became very turbid. There was, however, no flocculation, and the solutions, if removed from the bath, remained stable for an indefinite period.

*Heating control.* The solutions were heated in an open tinned copper bath containing glycerol. The bath was fitted with an efficient stirrer, and the temperature was controlled by a thermionic valve relay in conjunction with a contact thermometer (Baily, Grundy and Barrett). The temperature used throughout the entire series of experiments was 120° and did not vary by more than 0·25°.

As the experiments were carried out above boiling-point it was necessary to use closed tubes for the caseinogen solutions. A very cheap and efficient method was devised. Small heavy-walled pyrex tubes were used, each being closed with a rubber stopper. Each tube was fixed tightly in a metal clamp, a small piece of rubber being inserted as a cushion between the bottom of the tube and the clamp (see Fig. 1). This arrangement enabled the observer to shake, invert and remove each tube independently. After removal from the bath the tubes were cooled quickly in running water.

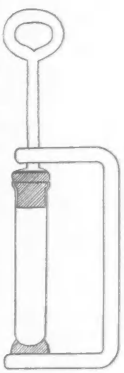


Fig. 1.

*Analysis.* Nitrogen determinations were made by the micro-Kjeldahl method. Phosphorus was estimated by the method of Fiske and Subbarrow [1925].

*Experimental procedure.* A number of tubes, each containing 5 ml. of 3·5 % sodium (or calcium) caseinogenate, were immersed in the glycerol-bath at 120° for periods of from 1 to 5 hours. The tubes were removed from the bath at appropriate intervals, cooled and, where necessary, shaken to break up any coagulum which had been formed. 5 ml. of 10 % trichloroacetic acid were then added to each tube. After standing for 5 minutes the contents were filtered through a No. 1 Whatman filter. Nitrogen and phosphorus determinations were made on aliquot portions of the filtrate. Each experiment was carried through in duplicate.

*Discussion of results.*

Typical curves showing the rate of liberation of acid-soluble phosphorus and nitrogen from a 3·5 % solution of sodium caseinogenate are given in Fig. 2. The equivalent values for a 3·5 % solution of calcium caseinogenate are given in Fig. 3.

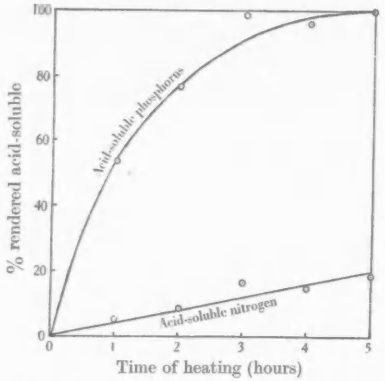



Fig. 2. Effect of heat on 3·5 % solution of sodium caseinogenate.

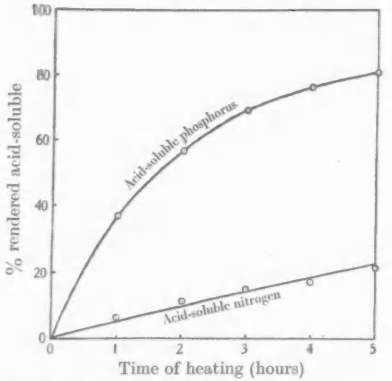



Fig. 3. Effect of heat on 3·5 % solution of calcium caseinogenate.

It will be seen that the general trend of the results is the same for both the sodium and calcium salts, there being a rapid liberation of acid-soluble phosphorus and a relatively slow liberation of acid-soluble nitrogen. Moreover, there appears to be no slowing off in the liberation of nitrogen when the entire phosphorus of the caseinogen has become acid-soluble. This result is comparable with that found by Rimington and Kay [1926] for mild alkaline hydrolysis but differs markedly from the findings of Stirling and Wishart [1932] in their study of the action of trypsin-kinase on caseinogen. The relatively small amount of nitrogen liberated by heat treatment, and the fact that (as shown in Figs. 2 and 3) nitrogen cleavage takes place at the same rate with both the sodium and calcium

salts, while the rate of phosphorus cleavage differs for these two salts, provide strong evidence that the effect of heating is to liberate phosphorus as inorganic phosphate and not in organic combination.

Rimington and Kay found that alkaline hydrolysis of caseinogen resulted in the production of acid-soluble nitrogen in the form of ammonia. In the present experiments no estimations of free ammonia were made, but the fact that the solutions tended to become more acid as heating proceeded precludes the formation of any considerable quantity of ammonia. It appears from these facts that the mode of liberation of nitrogen from the caseinogen molecule by heat treatment differs from that resulting from treatment with alkali.

As regards the rate of liberation of acid-soluble phosphorus, conditions were not sufficiently constant to allow for any final conclusion as to the order of the reaction, since there was invariably an increase in the acidity of the solution during heating. There is, however, one marked difference between the curves for the sodium and calcium salts. It will be seen that with the sodium salt, 100 % of the phosphorus is rendered acid-soluble within 5 hours, whereas with the calcium salt only 80 % is rendered acid-soluble. It appeared at first sight that this might be due to the fact that with the calcium salt a clot was formed within 2 hours, and that in spite of vigorous shaking part of the phosphorus might remain embedded in this clot. Such an explanation would not, however, account for the difference in the amounts of phosphorus which were rendered acid-soluble within the first hour (*i.e.* 54 and 37 % respectively) during which no coagulation occurred. Moreover, the form of the curve for the calcium salt indicates that the reaction is practically complete when only 80 % of the phosphorus is in the acid-soluble form. It seems possible that the explanation of this marked difference between the sodium and calcium salts lies in the catalytic effect of Na ions.

## II. The preparation and properties of heat-dephosphorised caseinogen.

### *Preparation of heat-dephosphorised caseinogen.*

One litre of 3·5 % sodium caseinogenate ($p_H$ 6·7) was prepared as previously described and placed in a 2 litre flask. The mouth of the flask was covered with a thin sheet of cellophane and the flask heated in an autoclave. In about 30 minutes the pressure had reached 1 atmosphere (121°) and it was retained at this value for 4 hours. A further 30 minutes was allowed for the pressure to fall, and the flask was then removed and cooled in running water. No coagulation occurred during the heating, but a thin skin was formed on the bottom of the flask. The heated solution was definitely pink in colour. The solution was then treated with small quantities of 33 % acetic acid until maximum precipitation took place [Rimington and Kay, 1926]. The gelatinous precipitate was filtered off through linen, well washed with water and redissolved in dilute NaOH. It was then reprecipitated with 33 % acetic acid. At this stage the precipitate appeared white, but it soon darkened to brown even though not directly exposed to air. After settling, decanting off the supernatant liquid and thorough washing, the precipitate was again filtered through linen and well drained. It was next transferred to a mortar and ground with absolute alcohol for about an hour. On first contact with alcohol the precipitate became a gummy mass, but with continued grinding it became a friable powder. This was filtered off on a Büchner funnel, re-extracted with fresh alcohol for another hour, again filtered off and finally allowed to dry in a current of air. The final product was

a brownish pink powder. The moisture content (mostly alcohol) was 15 %. This could be reduced by treatment with ether to about 10 %, but the product so obtained was less easily soluble in alkali; maximum yield, 49 %.

*Properties of heat-dephosphorised caseinogen.*

In order to compare the properties of the heat-dephosphorised caseinogen with those of the untreated caseinogen and of Rimington and Kay's alkali-dephosphorised caseinogen, samples of the latter two products were also prepared. The alkali-dephosphorised caseinogen was prepared according to Rimington and Kay's directions. The control sample of caseinogen was prepared from an untreated solution of 3·5 % sodium caseinogenate, the methods of precipitation and purification being identical with those used for the dephosphorised products. The nitrogen and phosphorus contents of the three preparations (expressed as % of dry matter) were as follows:

| | N (%) | P (%) |
|---|---|---|
| Original (untreated) caseinogen | 15·08 | 0·830 |
| Heat-dephosphorised caseinogen | 13·86 | 0·032 |
| Alkali-dephosphorised caseinogen | 13·56 | 0·016 |

Two methods were used for differentiating the three products, (*a*) the determination of their titration curves, and (*b*) the determination of their relative rates of coagulation when heated at 120°.

(*a*) *Titration curves.*

*Technique.* 1 % solutions (on dry matter basis) of each product were prepared. For acid-binding capacity the solutions were made up with standard HCl (approximately 0·1 *N*) of sufficient amount to give an initial $p_H$ of 2·0–3·0; for base-binding capacity sufficient standard NaOH (approximately 0·1 *N*) was used to give an initial $p_H$ of 8·0–9·0. The amount of acid or alkali added was noted. The method of determining the combining capacity was that of "back-titration" from the initial $p_H$ to the region of the isoelectric point. For this purpose known amounts of standard alkali or acid were added to aliquot portions of the original solutions, and the $p_H$ was determined after each addition.

For base-binding capacity the $p_H$ measurements were made with a glass electrode. For acid-combining capacity a quinhydrone electrode was used in combination with a saturated calomel half-cell. With both methods of determination the accuracy of the measurements was within 0·5 millivolt.

The method of calculation of the equivalents of acid or base bound was as follows:

*Acid-binding.* At any given $p_H$, equivalents bound per g. protein = [H] added − [H] found, where

[H] = concentration of acid in equivalents.

The concentration of acid added was obtained directly from the amount of HCl added *minus* the amount of NaOH added (expressed in equivalents). The concentration of free acid ([H] found) was calculated from the observed $p_H$.

*Base-binding.* At any given $p_H$, equivalents bound per g. protein = [OH] added − [OH] found, where

[OH] = concentration of alkali in equivalents.

The concentration of alkali added was obtained directly from the amount of NaOH added *minus* the amount of HCl added (expressed in equivalents). The concentration of free alkali ([OH] found) was calculated from the observed $p_H$.

The ionic product for water was taken as $K_w = 0{\cdot}74 \times 10^{-14}$ at 18° [Michaelis, 1926]. The degree of dissociation, $\alpha$, was taken as unity throughout the concentrations employed. Hoffmann and Gortner [1925] have pointed out that the degree of dissociation as determined by conductivity methods does not agree with the values obtained by potentiometric methods and suggest the use of the latter in calculating combining capacities. It was found that, up to the concentrations used in the present experiments, this correction was not of sufficient magnitude to be considered necessary.

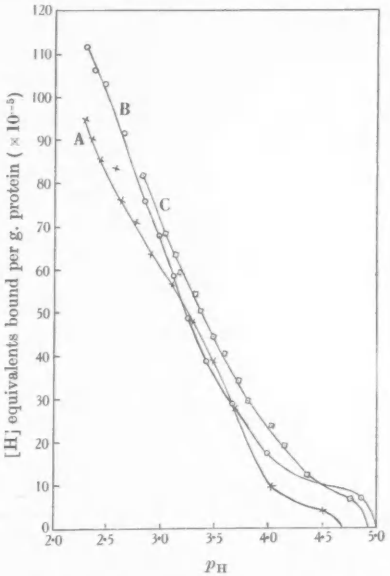



Fig. 4. Titration curves of *A*, untreated caseinogen; *B*, alkali-dephosphorised caseinogen; *C*, heat-dephosphorised caseinogen.

*Results.* The titration curves of the three products are shown in Figs. 4 and 5. The curves for the untreated caseinogen coincide fairly closely with those recorded by Pertzoff and Carpenter [1932]. It will be seen, however, that both alkaline hydrolysis and heat treatment have effected marked alterations in the base-binding capacity and, to a less extent, in the acid-binding capacity. Moreover, it is of special significance that the curves for the two dephosphorised products differ markedly, the alkali-dephosphorised caseinogen having a higher and the heat-dephosphorised caseinogen a lower base-binding capacity than untreated caseinogen. It has already been noted that the mode of liberation of phosphorus by heat appears to be identical with that caused by alkaline hydrolysis. On the other hand, it has been shown that the mode of cleavage of nitrogen probably differs in the two treatments. It seems likely therefore that the differences in combining capacity are associated with the cleavage of nitrogen rather than of phosphorus.

It should be added that, so far as the two dephosphorised products are concerned, it is not legitimate to consider these as specific proteins of fixed chemical

composition, as in the case of caseinogen. Owing to variations in the rate of heating and cooling of the autoclave, as well as to other conditions which cannot be kept exactly constant, two successive samples of the heat-dephosphorised caseinogen may vary somewhat in composition. The same criticism holds for the alkali-dephosphorised caseinogen. Such variations in composition are, however, unlikely to affect the acid- and base-binding capacities to any serious extent, and it is reasonable to assume that the curves shown in Figs. 4 and 5 represent typical acid- and base-binding capacities of the two types of dephosphorised product.

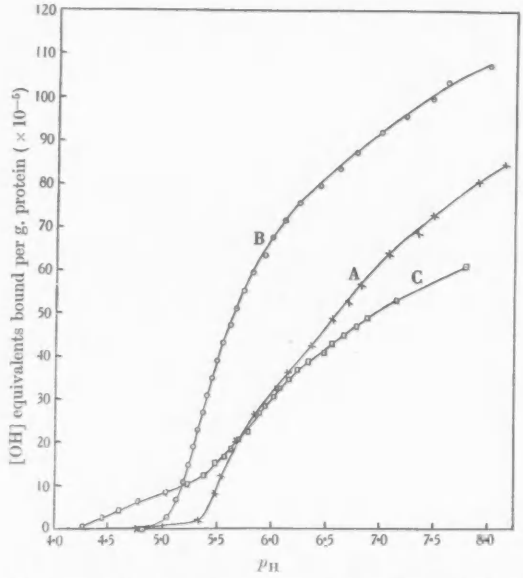



Fig. 5. Titration curves of A, untreated caseinogen; B, alkali-dephosphorised caseinogen; C, heat-dephosphorised caseinogen.

*(b) The rate of heat coagulation.*

*Technique.* 3 % solutions of the calcium salts of each of the three products already described were prepared as noted under (1) and (2) below. 5 ml. portions of the solutions were transferred to stoppered tubes, and the tubes were heated in a glycerol-bath at 120° (as previously described) for periods of from 1 to 5 hours. After removal from the bath the tubes were immediately cooled in water, the coagulated material was separated by centrifuging at 3000 r.p.m. for 5 minutes, and aliquot portions of the supernatant liquid were removed for analysis. Determinations were made of the total and non-protein nitrogen, the percentage of uncoagulated protein in the supernatant liquid being calculated by difference.

1. *Solutions containing minimum quantities of $Ca(OH)_2$.* The first series of experiments was carried out using the minimum quantities of $Ca(OH)_2$ necessary to give approximately neutral solutions. For this purpose the requisite amounts

of alkali were calculated from the titration curves already discussed (Fig. 5). The actual quantities of $Ca(OH)_2$ used, per 100 ml. of 3 % solution, were 0·061 g. for the untreated caseinogen; 0·051 g. for the heat-dephosphorised caseinogen; and 0·093 g. for the alkali-dephosphorised caseinogen. The $p_H$ values of the solutions varied from 6·83 to 6·99. The relative rates of coagulation of these solutions are shown (as unbroken lines) in Figs. 6, 7 and 8.

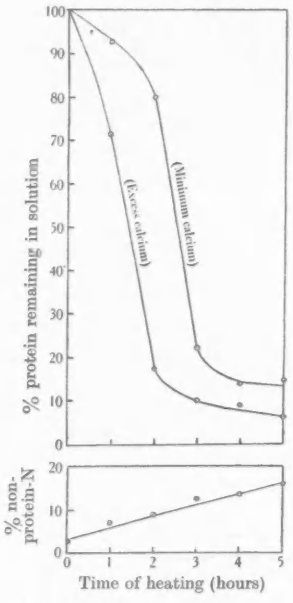



Fig. 6. Untreated caseinogen.

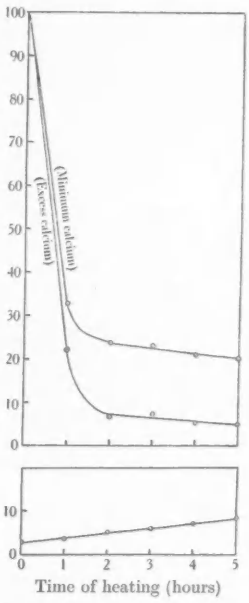



Fig. 7. Heat-dephosphorised caseinogen.

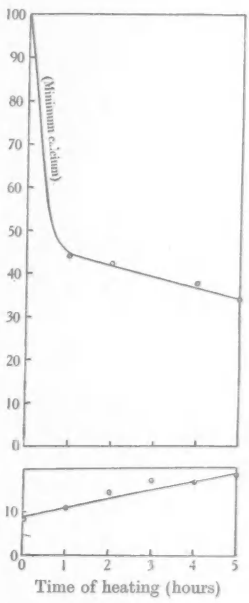



Fig. 8. Alkali-dephosphorised caseinogen.

It will be seen that the dephosphorised products coagulate very much more rapidly than the untreated caseinogen, and that the form of the coagulation curve obtained with the dephosphorised products differs fundamentally from that obtained with the caseinogen. Thus there is, during the first hour of heating, a rapid coagulation of the former products, over 70 % of the heat-dephosphorised caseinogen and nearly 60 % of the alkali-dephosphorised caseinogen being coagulated within this period. Within the same period barely 10 % of the untreated caseinogen is coagulated. After nearly 3 hours' heating, however, there is a sudden and marked increase in the rate of coagulation of the untreated caseinogen. If reference is made to Fig. 3, it will be seen that this increase coincides with the liberation of from 60 to 70 % of the phosphorus, so that, in that part of the curve where coagulation is most rapid, we are, in fact, dealing with a partly dephosphorised product, and not with untreated caseinogen.

The fact that the two dephosphorised products give coagulation curves of similar type, and that maximum coagulation occurs in the untreated caseinogen only after it has been partly dephosphorised, demonstrates the enhanced heat-

sensitivity which is associated with loss of phosphorus from the caseinogen molecule.

2. *Solutions containing excess Ca ions.* If reference is made to Fig. 5 it will be seen that the base-binding capacity of untreated caseinogen is higher than that of the heat-dephosphorised caseinogen. At $p_H$ 6·9 this difference amounts to nearly $10 \times 10^{-5}$ g.-eq. of alkali per 1 g. of protein. That is to say, the production of heat-dephosphorised caseinogen from a neutral solution of caseinogen will decrease the base-binding capacity by 16 %. It seemed reasonable to assume that this reduction in base-binding capacity would be accompanied (with the calcium salt, for example) by an actual liberation of calcium, either as free ions or in association with inorganic phosphate or protein degradation products. Now it has long been recognised that the presence of calcium ions tends to reduce the heat-stability of solutions of caseinogen, and it appeared possible, therefore, that such a liberation of calcium might be an important factor in accelerating coagulation of the heated caseinogen and/or of its dephosphorised product.

In order to investigate the effect of excess Ca ions on the rate of heat-coagulation of heat-dephosphorised caseinogen, a solution of the dephosphorised product was prepared which contained the same quantity of $Ca(OH)_2$ as was employed in making up the neutral solution of the untreated caseinogen (*i.e.* 0·061 g. $Ca(OH)_2$ per 100 ml. in place of 0·051 g. $Ca(OH)_2$). The $p_H$ of the solution was readjusted to neutral point by adding small quantities of 0·1 *N* HCl. The resultant solution can be considered as a solution of Ca caseinogenate in which the entire protein has been dephosphorised, but in which the original amount of calcium is still present. A similar additional quantity of $Ca(OH)_2$ was added to a solution of untreated Ca caseinogenate (and the $p_H$ adjusted with HCl) in order to investigate the effect of the excess Ca ions on the heat-coagulation of the unaltered caseinogen.

The relative rates of coagulation of these two solutions are shown in Figs. 6 and 7. It will be seen that the presence of excess Ca ions accelerates the coagulation of both solutions, the effect being more striking with the untreated caseinogen than with the dephosphorised product. The rate of coagulation of the untreated caseinogen, however, still lags behind that of the dephosphorised product throughout the greater part of the curve, the relative proportions coagulated at the end of one hour being 30 and 75 % respectively. Moreover, as has been pointed out in the previous section, the greater part of the caseinogen will have been dephosphorised within the first 2–3 hours of heating, so that in the latter half of the curve we are really dealing with the dephosphorised product and not with untreated caseinogen.

## Discussion and summary.

It is now possible to summarise in general terms the changes which result in, and accompany, the heat-coagulation of caseinogen solutions. If, for example, a neutral solution of calcium caseinogenate is heated at 120°, the following reactions will occur:

1. There will be a fairly rapid liberation of phosphorus from the caseinogen molecule, with the consequent conversion of the greater part of the caseinogen into a dephosphorised product. Concurrently a small part of the protein will be degraded, as shown by a progressive increase in the non-protein nitrogen fraction.

2. The dephosphorised product, being less heat-stable than the original caseinogen, will be rapidly coagulated by the continued heat treatment.

3. Since the base-binding capacity of the dephosphorised product is lower than that of caseinogen, its dephosphorisation will be accompanied by a liberation of calcium, either in ionic form or in association with inorganic phosphate or protein degradation products.

4. The excess Ca ions thus liberated will increase the heat-sensitivity of both the original caseinogen and the dephosphorised product, with consequent acceleration in the rate of coagulation.

It should be made clear that, while the two main reactions noted above, *i.e.* liberation of phosphorus and concurrent liberation of calcium, appear to play a large part in the heat-coagulation of solutions of calcium caseinogenate, they are probably not the only factors involved. For instance, changes in $p_H$ of the solution and alterations in the degree of dispersion and the degree of hydration of the protein particles will undoubtedly exert a considerable influence on the rate of heat-coagulation. Moreover, it must be borne in mind that the reactions described in this paper have been induced by abnormally drastic heat treatment, *i.e.* a temperature of 120° and heating for periods up to 5 hours. The results cannot, therefore, be directly applied to the type of heat coagulation which takes place in the manufacture of evaporated milks.

Nevertheless the results are of importance in that they provide an example of denaturation in which a distinct structural change, namely dephosphorisation of the molecule, forms a preliminary to coagulation. Such a finding appears to be without parallel in previous studies of denaturation and heat coagulation.

One of us (G. R. H.) is indebted to the Agricultural Research Council for the award of a Research Scholarship during the tenure of which this work was carried out.

## REFERENCES.

Chick and Martin (1912). *J. Physiol.* **43**, 1; **45**, 261.
Fiske and Subbarrow (1925). *J. Biol. Chem.* **66**, 375.
Hoffman and Gortner (1925). Colloid Symposium Monograph, **2**, 209.
Lewis (1926). *Biochem. J.* **20**, 965.
Michaelis (1926). Hydrogen ion concentration, **1**, 25 (English trans.).
Pertzoff and Carpenter (1932). *J. Gen. Physiol.* **16**, 257.
Rimington and Kay (1926). *Biochem. J.* **20**, 777.
Stirling and Wishart (1932). *Biochem. J.* **26**, 1989.

# CLXXXII. THE ABSORPTION OF MILK PRECURSORS BY THE MAMMARY GLAND.

## VI. THE RELATION OF PHOSPHORUS TO THE FAT METABOLISM OF LACTATION.

By JANET HENDERSON BLACKWOOD.

*From the Hannah Dairy Research Institute, Kirkhill, Ayr.*

(*Received June 30th, 1934.*)

In Part IV of this series [Blackwood and Stirling, 1932, 1] some consideration was given to certain aspects of the phosphorus metabolism of the mammary gland. While there were indications in the data obtained that the experimental proof of the phosphatide theory of fat secretion given by Meigs *et al.* [1919] could not be substantiated, it was felt that in the absence of lipin-phosphorus determinations such a conclusion was not then entirely justified. The present series of experiments was planned to explore more fully the postulated relationship between phosphorus in the blood and the fat metabolism of the mammary gland during lactation. At the same time it was thought desirable to determine the level of the total alcohol-ether-soluble fraction of the whole blood and plasma together with the cholesterol content during the passage of the blood through the active and inactive mammary gland of the cow.

### Experimental.

The technique of blood sampling was that employed in the earlier work of this series but it may be desirable to give our experience in detail. Samples of blood were drawn simultaneously from the radial artery and from the jugular and abdominal subcutaneous (mammary) veins of lactating and non-lactating cows. Arterial puncture in non-aesthetised cows is very frequently attended by disturbance. Where this seemed probable the animal in question was abandoned and a more suitable subject chosen. Finally it was found that by preliminary gauging of the size of the radial artery, it was possible to select animals for experiment such that disturbance was reduced to a minimum. The sample from the jugular vein was obtained with slight constriction either by finger pressure or by momentary application of a "choke" rope. In every case the entire process of jugular sampling was complete in about 30 seconds. No special measures were required for the mammary blood sample. No difficulty was ever experienced with either of the venous samples and no oedema resulted with this technique. Only those cases where the entire procedure of arterial, jugular and mammary sampling was completed within 4 minutes are included in the work on milk secretion. Each sample of 60 ml. volume was drawn into a dry sterile glass bottle containing 0·1 g. of sodium oxalate as anticoagulant. The plasma was separated immediately and the various fractions examined.

*Corpuscular volume.* The haematocrit technique was used. As each blood contained the same amount of anticoagulant the slight effect of the addition of sodium oxalate on corpuscular volume is eliminated.

*Total acid-soluble phosphorus and inorganic phosphate.* The colorimetric method of Fiske and Subbarow [1925] using certain of the modifications introduced by King [1932] was used, organic acid-soluble phosphorus being estimated by difference.

*Lipin-phosphorus.* The proteins of blood and plasma were precipitated by addition to a cold alcohol-ether mixture (3 parts of 97 % ethyl alcohol to 1 part of ether). After standing for 1½ hours the dilution was adjusted to 1 in 20 and the protein precipitate filtered off. Lipin-phosphorus was determined colorimetrically in the residue obtained by evaporating an aliquot of the alcohol-ether extract.

*Total alcohol-ether-soluble material.* An aliquot of the alcohol-ether filtrate for lipin-phosphorus determination was evaporated on the steam-bath and dried in an air-oven at 100° for 3 hours, and the residue was weighed after standing overnight in a desiccator.

*Cholesterol.* This was determined by Sackett's [1925] modification of Bloor's method using the Liebermann-Burchard reaction.

Results obtained with 12 animals, 6 lactating and 6 non-lactating, are reported in the following tables. The milk yield and yield of fat and phosphorus for each animal, together with its stage in lactation, are given in Table I only. Table VIII gives a list of the probable errors attaching to the individual determinations. The values of the various constituents in the corpuscles were also calculated from the haematocrit determinations, but since these are without significance they have been omitted from the tables.

## Discussion.

### *The phosphatide theory of fat secretion.*

The phosphatide theory of fat secretion as enunciated by Meigs *et al.* [1919] depends on the fact that if the mammary gland utilises the fatty acids of the phosphatide fraction of blood for the production of milk-fat, it will receive with the required amount of phosphatide-fatty acid more phosphorus than it requires for the secretion of the phosphorus compounds of milk. This excess phosphorus is presumed to be returned to the mammary venous blood where it serves to increase the level of inorganic phosphate. The removal of phosphatide by the gland will simultaneously result in a diminished content of lipin-phosphorus in the efferent blood. The work of Meigs *et al.* [1919] on jugular and mammary venous blood analyses appeared to give the experimental proof of this theory. It should be noted, however, that their results for non-lactating animals were rather conflicting, as there was a tendency for the differences found in the blood of lactating animals to be present in those non-lactating.

If reference is made to Tables I and II it will be seen that, so far as lactating animals are concerned, the mammary blood has a significantly lower lipin-phosphorus than the jugular blood, a fact which might at first sight be taken to support the phosphatide theory. It will be observed, however, that similar differences are found in the blood of non-lactating animals. The differences are, in fact, almost identical, being 4·3 % for the lactating and 4·2 % for the non-lactating animals. The differences in lipin-phosphorus content cannot therefore be attributed to lactation.

Table I. *Lipin-phosphorus. mg./100 ml.*

| Cow No. | Corpuscular volume: Jugular | Arterial | Mammary | Milk yield lb. per day | Fat g. per day | Phosphorus g. per day | Stage in lactation days | Blood: Jugular | Arterial | Mammary | Plasma: Jugular | Arterial | Mammary |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 33·0 | 29·0 | 30·0 | 39·5 | 642 | 17·1 | 36 | 11·03 | 10·60 | 10·37 | 8·04 | 7·40 | 7·70 |
| 1 | 41·7 | 39·0 | 39·6 | 17·5 | 254 | 6·3 | 93 | 9·90 | 9·60 | 9·86 | 5·87 | 5·72 | 5·72 |
| 2 | 33·3 | 31·0 | 34·7 | 25·0 | 438 | 10·0 | 122 | 9·78 | 9·40 | 9·35 | 6·43 | 6·08 | 6·10 |
| 3 | 34·0 | 32·5 | 32·2 | 21·5 | 343 | 9·9 | 157 | 10·97 | 10·28 | 10·23 | 7·22 | 7·06 | 7·13 |
| 3 | 38·7 | 32·0 | 34·9 | 16·5 | 306 | 8·5 | 185 | 9·53 | 9·12 | 9·08 | 6·61 | 5·97 | 6·35 |
| 4 | 33·5 | 31·6 | 32·7 | 15·5 | 277 | 6·1 | 146 | 9·72 | 9·45 | 9·37 | 6·40 | 6·30 | 6·30 |
| | | | | | | | Mean | **10·15** | **9·74** | **9·71** | **6·76** | **6·42** | **6·55** |
| | | | | Days dry | | | | | | | | | |
| 5 | 45·0 | 42·2 | 44·8 | 109 | | | | 9·92 | 9·38 | 9·75 | 5·74 | 5·69 | 5·96 |
| 6 | 40·0 | 38·0 | 39·5 | 100 | | | | 8·74 | 8·30 | 8·65 | 5·26 | 4·90 | 4·96 |
| 7 | — | — | — | 73 | | | | — | — | — | 4·26 | 4·38 | 4·37 |
| 8 | 29·4 | 31·0 | 29·7 | 45 | | | | 7·47 | 6·80 | 6·40 | — | — | — |
| 9 | — | — | — | 19 | | | | 9·22 | 8·95 | 8·93 | — | — | — |
| 10 | 43·6 | 42·0 | 42·0 | 13 | | | | 9·51 | 9·38 | 9·23 | 5·36 | 5·11 | 5·21 |
| | | | | | | | Mean | **8·97** | **8·56** | **8·59** | **5·15** | **5·02** | **5·12** |

Table II. *Differences in lipin-phosphorus. % arterial.*

| Cow No. | Date | Blood: Jugular-mammary | Arterial-jugular | Arterial-mammary | Plasma: Jugular-mammary | Arterial-jugular | Arterial-mammary |
|---|---|---|---|---|---|---|---|
| 1 | 21. xi. 33 | +6·23 | −4·06 | +2·17 | +4·60 | −8·65 | −4·05 |
| 1 | 17. i. 34 | +0·42 | −3·13 | −2·70 | +2·62 | −2·62 | 0 |
| 2 | 15. i. 34 | +4·58 | −4·04 | +0·53 | +5·43 | −5·76 | −0·33 |
| 3 | 16. xi. 33 | +7·22 | −6·71 | +0·49 | +1·27 | −2·27 | −0·99 |
| 3 | 14. xii. 33 | +4·94 | −4·50 | +0·44 | +4·36 | −10·72 | −6·35 |
| 4 | 29. xi. 33 | +3·71 | −2·86 | +0·85 | +1·59 | −1·59 | 0 |
| 5 | 23. i. 34 | +1·82 | −5·76 | −3·95 | −3·87 | −0·88 | −4·75 |
| 6 | 26. i. 34 | +1·08 | −5·30 | −4·22 | +6·13 | −7·35 | −1·23 |
| 7 | 7. xi. 33 | — | — | — | −2·51 | +2·74 | 0 |
| 8 | 13. xi. 33 | +15·75 | −9·85 | +5·88 | — | — | — |
| 9 | 27. xi. 33 | +3·25 | −3·02 | +0·22 | — | — | — |
| 10 | 28. iii. 34 | +2·98 | −1·39 | +1·60 | +2·94 | −4·89 | −1·96 |

It has been shown [Blackwood and Stirling, 1932, 2] that jugular blood is not truly representative of arterial blood in so far as its content of iron, glucose and amino-acids is concerned. These authors suggested that the concentration of blood-solutes due to the intervention of the salivary glands in the path of the blood entering the jugular vein, together with differences in the metabolic activities of the tissues drained by the jugular and abdominal subcutaneous veins, might be sufficient to invalidate comparisons of these two bloods for purposes of milk secretion investigations. In general, then, it is necessary that analytical values for mammary venous blood should be compared with those for arterial blood.

If such a comparison is made, reference to Tables I and II shows that in lactating animals there is no evidence of any consistent lowering of lipin-phosphorus during the passage of the blood through the mammary gland. Moreover, the phosphatide theory of fat secretion demands, as noted above, that there should be an abnormally high inorganic phosphorus content in the mammary blood. It will be seen from Tables III and IV that the inorganic phosphorus content of the mammary blood is, in fact, higher than that of the jugular blood. This is, however, again true for both lactating and non-lactating animals and cannot therefore be attributed to the process of milk secretion. If, on the other hand, the inorganic phosphorus content of the mammary blood

Table III. *P as inorganic phosphate. mg./100 ml.*

| Cow No. | Date | Blood | | | Plasma | | |
|---|---|---|---|---|---|---|---|
| | | Jugular | Arterial | Mammary | Jugular | Arterial | Mammary |
| 1 | 21. xi. 33 | 4·65 | 5·58 | 5·35 | 5·49 | 6·65 | 6·45 |
| 1 | 17. i. 34 | 5·28 | 5·67 | 5·47 | 6·29 | 6·85 | 6·60 |
| 2 | 15. i. 34 | 4·33 | 5·38 | 5·03 | 5·08 | 6·10 | 6·00 |
| 3 | 16. xi. 33 | 4·52 | 5·16 | 4·93 | 5·27 | 6·18 | 6·06 |
| 3 | 14. xii. 33 | 4·13 | 4·77 | 4·66 | 4·54 | 5·25 | 5·18 |
| 4 | 29. xi. 33 | 4·06 | 4·53 | 4·41 | 4·70 | 5·22 | 5·04 |
| | Mean | **4·49** | **5·18** | **4·97** | **5·23** | **6·04** | **5·89** |
| 5 | 23. i. 34 | 4·80 | 4·83 | 4·82 | 5·82 | 5·95 | 5·92 |
| 6 | 26. i. 34 | 5·28 | 5·39 | 5·40 | 6·43 | 6·58 | 6·63 |
| 7 | 7. xi. 33 | 4·15 | 4·32 | 4·39 | 4·86 | 5·00 | 5·18 |
| 8 | 13. xi. 33 | 5·31 | 5·29 | 5·29 | 6·10 | 6·19 | 6·03 |
| 9 | 27. xi. 33 | 4·61 | 4·99 | 5·10 | — | — | — |
| 10 | 28. iii. 34 | 4·79 | 5·00 | 4·98 | 5·95 | 6·29 | 6·26 |
| | Mean | **4·82** | **4·97** | **4·98** | **5·83** | **6·00** | **6·00** |

Table IV. *Differences in P as inorganic phosphate. % arterial.*

| Cow No. | Date | Blood | | | Plasma | | |
|---|---|---|---|---|---|---|---|
| | | Jugular-mammary | Arterial-jugular | Arterial-mammary | Jugular-mammary | Arterial-jugular | Arterial-mammary |
| 1 | 21. xi. 33 | −12·55 | +16·70 | +4·12 | −14·42 | +17·40 | +3·01 |
| 1 | 17. i. 34 | − 3·35 | + 6·90 | +3·53 | − 4·53 | + 8·18 | +3·65 |
| 2 | 15. i. 34 | −13·05 | +19·50 | +6·51 | −15·08 | +16·70 | +1·64 |
| 3 | 16. xi. 33 | − 7·95 | +12·40 | +4·46 | −12·80 | +14·70 | +1·94 |
| 3 | 14. xii. 33 | −11·10 | +13·40 | +2·31 | −12·20 | +13·50 | +1·33 |
| 4 | 29. xi. 33 | − 7·73 | +10·40 | +2·65 | − 6·52 | + 9·96 | +3·45 |
| 5 | 23. i. 34 | − 0·41 | + 0·60 | 0 | − 1·68 | + 2·19 | +0·50 |
| 6 | 26. i. 34 | − 2·23 | + 2·04 | 0 | − 3·04 | + 2·28 | −0·76 |
| 7 | 7. xi. 33 | − 5·55 | + 3·90 | −1·62 | − 6·41 | + 2·80 | −3·60 |
| 8 | 13. xi. 33 | + 0·38 | − 0·38 | 0 | + 1·13 | + 1·45 | +2·58 |
| 9 | 27. xi. 33 | − 9·84 | + 7·60 | −2·21 | — | — | — |
| 10 | 28. iii. 34 | − 3·81 | + 4·20 | +0·40 | − 4·94 | + 5·40 | +0·48 |

is compared with that of the artérial blood, it will be seen that with lactating animals there is a definite tendency for the inorganic phosphorus content of the mammary venous blood to be lower than that of arterial blood, and reference to the table of probable errors (Table VIII) shows that the difference is significant. With the non-lactating animals there is but slight variation in the inorganic phosphorus content of arterial and mammary venous bloods even though the physiological conditions of the animals are most diverse—some are seen to be approaching parturition, while others are only a few weeks or days removed from the last lactation. There is, moreover, no agreement in magnitude or direction in these slight differences. It may therefore reasonably be concluded that there is a small but significant diminution of inorganic phosphate in the efferent blood of the gland during lactation.

The results form a fairly conclusive demonstration that there is no basis for the assumption that phosphatide is the precursor of fat in the active mammary gland. Additional evidence is provided in the work of Sinclair [1931; 1932], who investigated the intermediary rôle assigned to the phospholipins in general fat metabolism. This worker claims, by the use of suitable diets, to have changed the iodine number of the fatty acids associated with tissue-phospholipins but suggests that the rate of turnover of these acids is not associated with the

intensity of fat metabolism. From these findings he concludes that the phospholipins present in muscles, and presumably those in the other tissues as well, should no longer be regarded as intermediary products in the metabolism of fats. In view, however, of the very great difficulties of proving experimentally that phosphatides play an intermediate rôle in fat metabolism, it is desirable that further evidence should be available before definite conclusions are drawn.

*The origin of the phosphorus in milk.*

It has been pointed out in the above discussion that there is a small but significant diminution of the inorganic phosphorus content of blood during its passage through the mammary gland. The diminution of inorganic phosphate in the efferent blood of the active gland is on the average 0·21 mg. of phosphorus per 100 ml. of blood. According to the figures published in Part II of this series the efferent blood contains 9·8 mg. less sugar than the arterial blood supply. Milk containing about 5 % of lactose and 0·1 % of phosphorus has about fifty times as much lactose as phosphorus. If the active gland requires 9·8 mg. of sugar to supply its needs, it might be expected that its requirement of phosphorus would be about one-fiftieth of this, or rather less than 0·2 mg. of phosphorus per 100 ml. of blood. There is therefore close agreement between the relative estimates of the requirements of the active mammary gland for sugar and phosphorus, and it may be tentatively concluded that the phosphorus of milk is derived from the inorganic phosphorus of the blood.

Table V. *Organic acid-soluble phosphorus. mg./100 ml.*

| Cow No. | Date | Blood | | | Plasma | | | Corpuscles | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Jugular | Arterial | Mammary | Jugular | Arterial | Mammary | Jugular | Arterial | Mammary |
| 1 | 21. xi. 33 | 2·81 | 2·92 | 3·09 | 0·00 | 0·05 | 0·02 | 9·10 | 10·00 | 10·03 |
| 1 | 17. i. 34 | 3·29 | 3·23 | 3·23 | 0·04 | 0·00 | 0·04 | 7·84 | 8·29 | 8·11 |
| 2 | 15. i. 34 | 2·52 | 2·22 | 2·58 | 0·00 | 0·17 | 0·11 | 7·57 | 6·80 | 7·24 |
| 3 | 16. xi. 33 | 2·47 | 2·56 | 2·50 | — | — | — | — | — | — |
| 3 | 14. xii. 33 | 2·85 | 2·43 | 2·59 | 0·68 | 0·61 | 0·66 | 6·29 | 6·31 | 6·18 |
| 4 | 29. xi. 33 | 2·54 | 2·46 | 2·46 | 0·40 | 0·32 | 0·36 | 6·78 | 7·09 | 6·80 |
| | Mean | **2·75** | **2·64** | **2·74** | **0·22** | **0·23** | **0·24** | **7·52** | **7·70** | **7·67** |
| 5 | 23. i. 34 | 2·79 | 2·67 | 2·47 | 0·31 | 0·32 | 0·24 | 5·83 | 5·91 | 5·22 |
| 6 | 26. i. 34 | 2·70 | 2·92 | 2·96 | 0·00 | 0·10 | 0·08 | 6·76 | 7·53 | 7·37 |
| 7 | 7. xi. 33 | 2·66 | 2·70 | 2·89 | 0·08 | 0·05 | 0·16 | — | — | — |
| 8 | 13. xi. 33 | 1·01 | 1·67 | 2·33 | — | — | — | — | — | — |
| 9 | 27. xi. 33 | 2·95 | 3·19 | 3·16 | — | — | — | — | — | — |
| 10 | 28. iii. 34 | 2·75 | 3·14 | 2·92 | 0·00 | 0·10 | 0·07 | 6·31 | 7·35 | 6·81 |
| | Mean | **2·48** | **2·71** | **2·79** | **0·10** | **0·14** | **0·14** | **6·30** | **6·93** | **6·46** |

Basing their conclusions on the values obtained in the earlier series of experiments Blackwood and Stirling suggested that the active mammary gland withdraws organic acid-soluble phosphorus compounds from the blood-stream and that such compounds might be concerned in the secretion of colloidal calcium phosphate, thus bringing milk secretion into line with bone formation. Statistical examination of the figures given in support of the above suggestion has since shown, however, that such an interpretation cannot be put upon the figures with any degree of certainty. Moreover, the determination of organic acid-soluble phosphorus in plasma cannot be made with the same degree of accuracy as that of the other phosphorus fractions, and conclusions based on its variation in the plasma must be tentative.

The values now reported for organic acid-soluble phosphorus are, in fact, lower than those of the previous series. They are, however, in fairly good agree-

ment with those of McCay and Maynard [1931] and McCay [1931]. The most significant difference is in the extremely small amounts of organic acid-soluble phosphorus found in the plasma and in the considerable diminution of these compounds in the whole blood in the present series. As a check on these values 6 animals were taken from neighbouring herds, 3 non-lactating and 3 lactating. The latter gave 5 gallons of milk per day. Mammary venous blood was taken from each and analysed in the usual way for total acid-soluble phosphorus and inorganic phosphorus in whole blood and plasma. The results are shown in Table IX.

Again the amount of organic acid-soluble phosphorus compounds in the plasma is seen to be almost negligible, so that differences in feeding and management of the animals are not responsible for the lower values now reported. There were, however, considerable differences in the ages of the animals in the two series, the Institute herd in 1931 consisting mainly of animals in their first lactation. No animal in the first series was less than 3 years or more than 4 years old, whereas the ages in the present series vary from 4 to 9 years with an average age of 6 years. The growth of the cow, although slow after the first 3 years, cannot be said to be complete until the age of 5 years and it seemed possible that this factor might influence the composition of the blood. A series of determinations of the phosphorus distribution in the blood and plasma at various age levels did not, however, bring out any difference sufficiently marked to give rise to a general age level gradient, and these values have been omitted from the tables.

The poverty of cow's blood in organic acid-soluble phosphorus compounds as compared with that of other species is again most marked, the content being about one-eighth of that of rabbit's blood. The greater part of the acid-soluble organic phosphorus of the blood in most species is carried in the red cells, and in this respect, as reference to Table V shows, the present values for the blood of cows are in line with the general experience.

*The origin of the milk fat.*

If phospholipin may no longer be presumed to act as sole intermediary in the fat metabolism of the mammary gland, it is of interest to consider the values of the other blood lipoids during lactation. Reference to Table VI shows that there are no significant differences in total lipoids between arterial and mammary venous blood and plasma in the lactating state within the limits of the experimental procedure employed. With non-lactating animals there is a tendency towards larger variations and a lack of uniformity which may be attributed to the varying stages in pregnancy of the animals (see below). Blood-cholesterol (Table VII) at first sight appears to be somewhat reduced on passage through the active gland, but the differences are inconsistent and the results, particularly with non-lactating animals, too variable to be correlated with functional activity.

If the mean values for the various lipoid fractions in the lactating and non-lactating states are compared, it is seen that lactation demands a high level of cholesterol and phospholipins in the blood and plasma generally. As lactation proceeds these values tend to fall off until, in the dry period before parturition, the level of all lipoids is considerably reduced. This is in harmony with the work of Maynard *et al.* [1931], who, in their study of the influence of the lactation cycle on the fatty acids, phosphatide-fatty acids and cholesterol of the blood, found that, following parturition, there was a rapid and approximately similar rise in all these constituents. This rise was succeeded by a gradual fall

Table VI. *Total alcohol-ether-soluble extract. g./100 ml.*

| Cow No. | Date | Blood | | | Plasma | | | Differences in blood % arterial | | | Differences in plasma % arterial | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Jugular | Arterial | Mammary | Jugular | Arterial | Mammary | Jugular-mammary | Arterial-jugular | Arterial-mammary | Jugular-mammary | Arterial-jugular | Arterial-mammary |
| 1 | 21. xi. 33 | 1·168 | 1·172 | 1·172 | 1·280 | 1·260 | 1·240 | −0·23 | +0·23 | 0 | +3·18 | −1·59 | +1·59 |
| 1 | 17. i. 34 | 1·040 | 1·032 | 1·020 | 1·112 | 1·156 | 1·080 | +1·93 | −0·78 | +1·16 | +2·77 | +3·81 | +6·57 |
| 2 | 15. i. 34 | 1·052 | 1·068 | 1·056 | 1·180 | 1·172 | 1·164 | −0·37 | +1·50 | +1·12 | +1·36 | −0·68 | +0·68 |
| 3 | 16. xi. 33 | 1·120 | 1·100 | 1·092 | 1·164 | 1·202 | 1·192 | +2·55 | −1·82 | +0·73 | −2·33 | +3·16 | +0·83 |
| 3 | 14. xii. 33 | 1·068 | 1·052 | 1·060 | 1·164 | 1·132 | 1·188 | +0·76 | −1·52 | −0·76 | −2·12 | −2·83 | −4·95 |
| 4 | 29. xi. 33 | 1·084 | 1·096 | 1·088 | 1·132 | 1·148 | 1·108 | −0·37 | +1·09 | +0·73 | +2·09 | +1·39 | +3·48 |
| | Mean | **1·089** | **1·087** | **1·081** | **1·172** | **1·178** | **1·162** | | | | | | |
| 5 | 23. i. 34 | 1·008 | 1·036 | 1·008 | 1·124 | 1·124 | 1·124 | 0 | +2·70 | +2·70 | 0 | 0 | 0 |
| 6 | 26. i. 34 | 1·028 | 1·032 | 1·004 | 1·048 | 1·032 | 1·068 | +2·33 | +0·39 | +2·71 | −1·94 | −1·55 | −3·49 |
| 7 | 7. xi. 33 | 0·992 | 1·060 | 1·012 | 1·088 | 1·060 | 1·100 | −1·89 | +6·40 | +4·53 | −1·13 | −2·64 | −3·77 |
| 8 | 13. xi. 33 | 1·016 | 1·040 | 0·992 | 1·044 | 1·068 | 1·036 | +2·31 | +2·31 | +4·62 | +0·75 | +2·25 | +2·99 |
| 9 | 27. xi. 33 | 1·004 | 1·040 | 1·036 | — | — | — | −3·08 | +3·46 | +0·38 | — | — | — |
| 10 | 28. iii. 34 | 1·016 | 1·020 | 1·032 | 1·068 | — | 1·080 | −1·57 | +0·39 | −1·07 | −1·12 | — | — |
| | Mean | **1·011** | **1·038** | **1·014** | **1·074** | **1·071** | **1·082** | | | | | | |

Table VII. *Cholesterol. mg./100 ml.*

| Cow No. | Date | Blood | | | Plasma | | | Differences in blood % arterial | | | Differences in plasma % arterial | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Jugular | Arterial | Mammary | Jugular | Arterial | Mammary | Jugular-mammary | Arterial-jugular | Arterial-mammary | Jugular-mammary | Arterial-jugular | Arterial-mammary |
| 1 | 21. xi. 33 | 214·6 | 211·5 | 204·4 | 220·8 | 207·3 | 196·8 | +4·83 | −1·47 | +3·36 | +11·60 | −6·51 | +5·06 |
| 1 | 17. i. 34 | 160·2 | 167·2 | 167·0 | 136·3 | 131·6 | 129·0 | −4·07 | +4·19 | 0 | +5·60 | −3·57 | +1·98 |
| 2 | 15. i. 34 | — | 197·7 | 194·6 | 193·2 | 190·4 | 196·3 | — | — | +1·57 | −1·63 | −1·47 | −3·10 |
| 3 | 16. xi. 33 | 187·8 | 181·8 | 175·4 | 185·4 | 206·1 | 188·5 | +6·83 | −3·30 | +3·52 | −1·51 | +10·00 | +8·54 |
| 3 | 14. xii. 33 | 151·8 | 171·2 | 159·7 | 155·9 | 150·5 | 148·3 | −4·62 | +11·33 | +6·72 | +5·06 | −3·59 | +1·46 |
| 4 | 29. xi. 33 | 161·5 | 175·0 | 171·8 | 148·1 | 147·6 | 143·3 | −5·89 | +7·71 | +1·83 | +3·26 | −0·34 | +2·91 |
| | Mean | **175·0** | **184·0** | **179·0** | **173·0** | **172·0** | **167·0** | | | | | | |
| 5 | 23. i. 34 | 166·7 | 162·0 | 154·6 | 158·8 | 156·5 | 156·1 | +7·47 | −2·90 | +4·57 | +1·73 | −1·47 | +0·26 |
| 6 | 26. i. 34 | 147·8 | 153·5 | 152·6 | 132·9 | 120·1 | 120·6 | −3·13 | +3·71 | +0·59 | +10·20 | −10·66 | −0·42 |
| 7 | 7. xi. 33 | 159·5 | 167·2 | 182·4 | 120·5 | 123·9 | 116·2 | −13·70 | +4·61 | −9·09 | +3·50 | +2·74 | +6·21 |
| 8 | 13. xi. 33 | 110·2 | 123·7 | 128·4 | 134·4 | 147·3 | 122·7 | −14·70 | +10·91 | −3·79 | +7·90 | +8·76 | +16·70 |
| 9 | 27. xi. 33 | 224·0 | 235·4 | 205·0 | — | — | — | +8·08 | +4·84 | +12·91 | — | — | — |
| 10 | 28. iii. 34 | 147·0 | 140·6 | 134·4 | 118·0 | 124·7 | 109·2 | +8·97 | −4·56 | +4·41 | +7·1 | +5·40 | +12·40 |
| | Mean | **159·0** | **164·0** | **160·0** | **133·0** | **134·0** | **125·0** | | | | | | |

Table VIII. *Standard deviations.*

| | Analyses | | | Mean | |
|---|---|---|---|---|---|
| Total alcohol-ether-soluble extract, g./100 ml. blood | (i) 1·036 | 1·052 | 1·060 | 1·049 | 0·010 |
| | (ii) 1·120 | 1·136 | 1·152 | 1·136 | 0·013 |
| | (iii) 1·100 | 1·108 | 1·112 | 1·107 | 0·005 |
| Cholesterol, mg./100 ml. blood | 205·0 | 205·0 | 208·7 | 206·2 | 1·740 |
| Lipin-phosphorus, mg./100 ml. blood | 8·93 | 9·13 | 8·92 | 8·96 | 0·102 |
| Inorganic phosphorus, mg./100 ml. blood | 5·10 | 5·09 | 5·11 | 5·10 | 0·0081 |
| Total acid-soluble phosphorus, mg./100 ml. blood | 8·26 | 8·24 | 8·23 | 8·24 | 0·013 |

Table IX.

| | Corpuscular volume | Blood | | | Plasma | | |
|---|---|---|---|---|---|---|---|
| Cow | % | Total acid-sol. P | Inorg. P | Organic acid-sol. P | Total acid-sol. P | Inorg. P | Organic acid-sol. P |
| Lactating | 32·5 | 7·01 | 4·33 | 2·68 | 5·10 | 4·97 | 0·13 |
| ,, | 33·0 | 6·51 | 4·32 | 2·19 | 4·90 | 4·92 | 0·00 |
| ,, | 32·5 | 6·86 | 4·73 | 2·13 | 5·22 | 5·27 | 0·00 |
| | Mean | **6·79** | **4·46** | **2·33** | **5·07** | **5·05** | |
| Non-lactating | 37·5 | 7·49 | 4·68 | 3·21 | 5·74 | 5·55 | 0·19 |
| ,, | 32·5 | 8·85 | 6·47 | 2·38 | 7·17 | 7·22 | 0·00 |
| ,, | 29·0 | 6·96 | 4·52 | 2·44 | 5·17 | 5·15 | 0·00 |
| | Mean | **7·77** | **5·22** | **2·68** | **6·03** | **5·97** | |

to normal levels as lactation advanced. The experience of Schaible [1932] was somewhat similar. In addition he was unable to demonstrate any change following parturition and throughout lactation in the distribution of the fatty acids between the blood-fat components or in their nature as determined by the degree of unsaturation. McCay and Maynard [1931] claim that the phospholipin and total phosphorus contents of blood-plasma depend on the level of dietary fat, although no phosphorus compounds other than the plasma-phosphatides are affected. A ration low in fat will lower the milk yield but does not affect the fat percentage [Maynard and McCay, 1929]. The level of the various lipoids in the blood-plasma has also been shown to be affected by changes in the quantity of the fat fed, while the iodine value of the total lipoids of the plasma may vary with the nature of the dietary fat [Williams and Maynard, 1934]. It is interesting to note also that the researches of Fürth and Scholl [1930], Szörenyi [1932] and Müller [1932] on the influence of lecithin on the solubility of fatty acids in bile-acid solutions have led to the conclusion by the latter that lecithin increases the dispersion of fatty acids but does not render them more diffusible.

While a considerable amount of work has been carried out in the field of mammary fat metabolism substantial progress is hindered by the lack of reliable methods for the separation and determination of the various fatty constituents of blood. It should also be borne in mind that the mammary gland may and probably does utilise more than one constituent of the blood as precursor in the synthesis of milk-fat. In addition, the permeability of the membrane of the secreting cells has not been fully studied, so that *a priori* theories cannot be subjected to sufficiently rigorous examination. It can only be concluded therefore that the mechanism of fatty acid transportation across the cell membranes of the mammary gland and the nature of fat synthesis in the secreting cells are still obscure.

SUMMARY.

The rôle of blood-phosphatide as the precursor of milk-fat has been investigated. The evidence adduced in favour of this theory has been shown to be incorrect when the relevant comparison of arterial and mammary venous blood is made.

There is close agreement between the relative estimates of the requirements of the active mammary gland for sugar and phosphorus.

Lactation demands a high level of all blood-lipoids, while the progress of lactation is accompanied by a fall to lower levels, which is most marked when the non-lactating period intervenes.

I am greatly indebted to Dr N. C. Wright for his help and encouragement throughout this work and also to Dr S. Dawson for his valuable criticism.

It is a pleasure to acknowledge the interest and assistance of Dr L. Jordan and Mr A. B. Fowler who obtained the blood samples.

REFERENCES.

Blackwood and Stirling (1932, 1). *Biochem. J.* **26**, 778.
—— —— (1932, 2). *Biochem. J.* **26**, 357, 362, 772, 778.
Fiske and Subbarow (1925). *J. Biol. Chem.* **66**, 375.
Fürth and Scholl (1930). *Biochem. Z.* **222**, 430.
King (1932). *Biochem. J.* **26**, 292.
Maynard, Harrison and McCay (1931). *J. Biol. Chem.* **92**, 263.
—— and McCay (1929). *J. Nutrition*, **11**, 67.
McCay (1931). *J. Biol. Chem.* **90**, 497.
—— and Maynard (1931). *J. Biol. Chem.* **92**, 273.
Meigs, Blatherwick and Cary (1919). *J. Biol. Chem.* **37**, 1.
Müller (1932). *Biochem. Z.* **249**, 189.
Sackett (1925). *J. Biol. Chem.* **64**, 203.
Schaible (1932). *J. Biol. Chem.* **95**, 79.
Sinclair (1931). *J. Biol. Chem.* **92**, 245.
—— (1932). *J. Biol. Chem.* **95**, 393.
Szörenyi (1932). *Biochem. Z.* **249**, 182.
Williams and Maynard (1934). *J. Dairy Sci.* **17**, 223.

*Note added August 20th, 1934.* Since this work was submitted for publication a paper by Lintzel (1934), *Z. Züchtg.: B. Tierzüchtg. Züchtgsbiol.* **29**, 219, dealing with the same subject has come to hand. The results therein reported are in substantial agreement with the present work and also with the earlier papers of this series.

# CLXXXIII. A DEFICIENCY DISEASE IN CHICKS RESEMBLING SCURVY.

By HENRIK DAM and FRITZ SCHÖNHEYDER.

*From the Biochemical Institute, University of Copenhagen.*

(*Received June 15th, 1934.*)

During work on sterol metabolism in the chick [Dam, 1929; 1930] it was observed that chicks reared on an artificial diet consisting of vitamin A-free caseinogen 20 parts, marmite 10, soluble starch 65·5, salts [Hart *et al.*, 1920] 4·5+ varying amounts of cod-liver oil concentrates had a marked tendency to large subcutaneous and intramuscular haemorrhages and certain pathological changes in the stomach. The growth was subnormal.

It has later been shown that in the original diet the marmite may be replaced by 15 % dried brewer's yeast, the soluble starch by rice starch or sucrose, the salts by other salt mixtures (*e.g.* the special salt mixture described below), while cod-liver oil may be used instead of cod-liver oil concentrates. The low content of sterol and fat was of no importance, because the addition of cholesterol and cod-liver oil did not prevent the symptoms.

It was originally supposed that the disease was due to the lack of vitamin C in the diet, in spite of the fact that other workers have found that chicks do not need vitamin C [Plimmer and Rosedale, 1923; Hart *et al.*, 1924; 1925]. Additions of lemon juice up to 77 ml. per animal in 59 days were, however, without the slightest influence on the occurrence of the symptoms [Dam, 1930], and an attempt has now been made to give pure ascorbic acid *per os* or subcutaneously even in very large doses. The effect, however, has been absolutely negative.

When the starch in the artificial diet is replaced by a mixture of cereals, the symptoms never occur. Cereals must therefore contain a factor which prevents the tendency to haemorrhages and which is different from vitamin C.

In their work on the fat-soluble vitamin requirements of the chick, McFarlane *et al.* [1931] observed haemorrhages resembling those described in this paper but closely associated with loss of power of clotting. These authors did not observe haemorrhages, when the protein of the diet was caseinogen which had been extracted with ether. When ether-extracted white fish meal or ether-extracted meat powder was used in lieu of caseinogen the haemorrhages occurred. Rations containing fish meal and meat powder which had not been extracted with ether did not cause haemorrhages.

### *Description of the symptoms*[1].

*Haemorrhages.* The haemorrhages have been observed in 60–70 % of the chicks reared on the basal diet. They set in after 11 days or later, most frequently after 15–20 days.

[1] This description is based on the autopsy of several hundred chicks of which only a small number had been used in the ascorbic acid experiments referred to in this paper.

There is no doubt that there is an important traumatic factor in the development of the haemorrhages. This explains their frequent occurrence in the left side of the breast where injections of ascorbic acid or NaCl have been given. Intramuscular and subfascial haemorrhages have been observed after pinching the muscles on the femur. Cutaneous haemorrhages occurred only in very exceptional cases. The subcutaneous haemorrhages were of different sizes varying from about $\frac{1}{2}$ cm.$^2$ up to the whole surface of the truncus.

In the majority of the cases the haemorrhages were found on breast, legs and wings. Intramuscular haemorrhages were particularly prone to occur on the legs. There was no predilection region about the knee joints. Intra-articular haemorrhages occurred only in a few cases. Sub-periostal haemorrhages have never been seen. In some cases large haemorrhages in the abdominal cavity were found and, rather seldom, petechial haemorrhages in the liver. One chick had a large retrobulbary haemotoma. The large haemorrhages began suddenly and were followed by severe anaemia. Whether the anaemia was a mere consequence of the loss of blood has not yet been established. External haemorrhages were practically never observed.

The haemorrhages were often absorbed in the course of a few days and in some cases the animals could be reared up to the age of 2 months, at which time the experiments were interrupted.

A few animals had very large oedemas.

*Changes in the mucous membrane of the stomach.* The stomach of the chick consists of two parts: the cardiac cavity and the gizzard, separated from each other by an intermediary zone. At autopsy the stomach was always opened in the posterior left side by a section through the posterior main muscle in order to obtain a good survey of the inside of the gizzard. On the frontal left side and the right rear surface the secretory lining is uniformly striated, and on the upper part surrounding the orifice of the duodenum the wall of the gizzard is thin and is formed from the fibres of the musculus intermedius. The surface of the mucosa of this part shows a finely mammelated design.

Apart from very rare cases of hyperplastic inflammation in the cardiac cavity this part of the stomach never showed any pathological change. Changes were, however, constantly found in the mucous membrane of the gizzard. Macroscopically the changes appear as more or less brown coloured areas where the lining is irregularly granulated and frayed. The colour is due to haemoglobin decomposition products. The size may vary from small spots to more than 1 cm.$^2$ Small lesions often resemble tumours projecting from the surface, but ulcerated areas have also been seen. The haemorrhagic floor of the lesions may be seen from the outside when they occur on the thin-walled part of the gizzard. When large lesions occur, the contents of the gizzard may be tar-coloured and give a positive phenolphthalin reaction. Instead of the uniformly striated surface already described the surface generally was found coarsely mammelated.

The affected areas are most frequently found on the upper part of the rear wall corresponding to the musculus intermedius superior but may occur on the whole surface.

Such macroscopic changes have been found after 16 days in nearly 100 % of the chicks which were reared on the basal diets 49 and 60 and in about 50 % after 11 days.

Microscopical examinations of the affected areas have so far revealed the following facts. While the normal secretory lining is almost homogeneous so that it is somewhat difficult to see how the secretions of the individual tubular glands continue up to the surface of the lining, with arch-shaped interconnec-

tions including traces of nucleus-containing substance, the lining in the affected areas clearly reveals its structure, is less compact and has large semilunar cavities containing collections of erythrocytes. At some places the lining has quite disappeared. The haemorrhagic infiltration of the secretion may sometimes be observed in the lumen of the glands, which may be stuffed with erythrocytes in the fundus. The tunica proprica itself also shows characteristic changes. The fundus of the glands is often dilated and the glandular epithelium has undergone a change which converts it from a typical cylindrical epithelium with basally placed nuclei to a shallow cubical epithelium with central nuclei.

The interglandular tissue is not much altered. It may be somewhat atrophic when the glands are strongly dilated. Signs of infiltration in the form of leucocyte-infiltration under surface epithelium very seldom occur. Hyperaemia or oedema has not been observed. Only in one single case signs of haemorrhages in the submucosa were found (a haemorrhage in process of absorption). The affections may not be considered therefore as ulcerations or inflammations in the proper sense.

The changes in the lining, the haemorrhages and the atrophic changes in the glands dominate the microscopic picture. The relationships between these symptoms will be investigated further. These conditions are illustrated by Figs. 1–4, Plate VIII.

## Experimental.

The birds were day-old White Leghorns. They were kept indoors in cages with wire screen bottoms in order to prevent coprophagy. No food was given in the first 24 hours after hatching. Food and tap-water could be taken *ad libitum*. Injections and additions by pipette were not given before the 4th day after hatching. At autopsy all organs were macroscopically inspected, and in a large number of cases microscopic preparations (paraffin technique) were made, particularly from the gizzards.

*Groups 49–53.* Basal diet "49." Caseinogen, vitamin A-free, 20 parts, dried yeast 15, sucrose 60, salts (Hart, Halpin and Steenbock) 5, cod-liver oil 4 (Norwegian cod-liver oil which according to test in the vitamin laboratory of the Norwegian State contains at least 500 A and 250 D Norwegian units per g.).

*Additions. Group 49* subcutaneous injection of ascorbic acid, brought with $NaHCO_3$ to weak acid reaction and made isotonic with NaCl. Initial dose per animal per day 0·32 mg. ascorbic acid, the daily dose being increased from week to week. The solutions were made every second day with oxygen-free water and stored in the ice-box. The amount of liquid injected varied from 0·05 to 0·1 ml., the solution being made more concentrated according to the age of the animals.

*Group 50.* No addition.

*Group 51.* Ascorbic acid (almost neutralised) by mouth by means of a pipette. The solution consisted of ascorbic acid 30 mg., $NaHCO_3$ 13 mg., water 1 ml., prepared with the same precautions as in group 49. Initial dose 1·5 mg. per animal per day.

*Group 52.* Ascorbic acid, not neutralised, subcutaneous. The solution consisted of ascorbic acid 30 mg., NaCl 45 mg., water 1 ml. Initial dose 1·5 mg.

*Group 53.* Ascorbic acid subcutaneous. The same solution as in group 51. Initial dose 1·5 mg.

The ascorbic acid was procured from Merck, Darmstadt. By titration against 0·001 *N* 2:6-dichlorophenolindophenol and against 0·005 *N* NaOH it was shown to contain 95–98 % of the pure substance.

*Group 60.* Diet: caseinogen, vitamin A-free, 20 parts, dried yeast 15, salts No. 2[1] 2·7, sucrose 62·7, cod-liver oil 4.

Subcutaneous injections of 0·9 % NaCl were given daily.

*Group 66.* Diet: the sucrose in diet 60 was replaced by a mixture of cereals and seeds commonly used as a constituent of ordinary chicken rations: caseinogen, vitamin A-free, 20 parts, dried yeast 15, salts No. 2 2·7, $CaCO_3$ 0·3, powdered mixture[2] 62, cod-liver oil 4.

Subcutaneous injections of 0·9 % NaCl daily.

| Chick No. | Age in days when dead | killed | Weight g. | Ascorbic acid mg. | Haemorrhages | Pathol. changes in the gizzard |
|---|---|---|---|---|---|---|
| *Group 49* | | | | | | |
| 406 | — | 24 | 83 | 10·7 | In subcutaneous tissue on abdomen | + + |
| 407 | — | 20 | 71 | 6·8 | Subcut. on right femur, subcut. and subfascial on the neck | . |
| 408 | — | 24 | 97 | 10·7 | Subfascial over right knee | + |
| 409 | — | 18 | 48 | 5·6 | 0 | + |
| 410 | — | 27 | 36 | 14·4 | Right side of breast | + |
| *Group 50* | | | | | | |
| 411 | — | 33 | 57 | 0 | Subfascial right elbow | + + |
| 412 | — | 31 | 64 | 0 | 0 | + + |
| 413 | — | 13 | 39 | 0 | 0 | + |
| 414 | — | 23 | 70 | 0 | Right wrist, in the joint, intramuscular outside of right leg | + + |
| 415 | — | 25 | 50 | 0 | 0 | + |
| *Group 51* | | | | | | |
| 416 | — | 20 | 57 | 35·7 | Intramus. on right shoulder and right humerus | + |
| 417 | — | 25 | 62 | 49·8 | Subcut. right middle foot | + + |
| 418 | — | 18 | 70 | 29·7 | Intramus. left leg | + + |
| 419 | — | 19 | 49 | 32·7 | Intramus. breast, right shoulder | + + |
| 420 | — | 24 | 90 | 49·8 | Subcut. left radio carp. | + + |
| *Group 52* | | | | | | |
| 421 | — | 19 | 51 | 31·8 | Large intraperitoneal | + + |
| 422 | 17 | — | 40 | 29·1 | Large intraperitoneal | + + |
| 423 | — | 24 | 51 | 52·2 | Subcut. and intram. left side of breast | + + |
| 424 | — | 25 | 44 | 55·8 | Petechial in liver | + |
| 425 | — | 13 | 54 | 19·0 | Intramus. left side of breast | + |
| *Group 53* | | | | | | |
| 426 | 14 | — | 39 | 19·2 | Large intraperitoneal | + |
| 427 | — | 13 | 37 | 16·8 | Large intraperitoneal, right side | + |
| 428 | — | 7 | . | . | 0 | . |
| 429 | — | 20 | 82 | 32·7 | Intramus. right leg and left side of breast | + + |
| 430 | — | 18 | 65 | 27·0 | Intramus. right side of breast | + + |
| *Group 60* | | | | | | |
| 471 | — | 20 | 82 | 0 | Subcut. right breast, abdomen, petechial in liver | + |
| 472 | — | 19 | 105 | 0 | Intramus. and subcut. on legs | + + |
| 473 | — | 25 | 70 | 0 | 0 | + + |
| 474 | | 18 | 69 | 0 | Subcut. right wing, leg, abdom. | + |
| 475 | 16 | — | 64 | 0 | Intraperitoneal left side | + + |
| *Group 66* | | | | | | |
| 502 | — | 58 | 625 | 0 | 0 | 0 |
| 503 | — | 56 | 550 | 0 | 0 | 0 |
| 504 | — | 58 | 577 | 0 | 0 | 0 |
| 505 | — | 58 | 604 | 0 | 0 | 0 |
| 506 | — | 58 | 548 | 0 | 0 | 0 |

[1] $CaCO_3$, 2·00; $MgCO_3$, 0·10; Ferric citrate, 0·16; NaCl, 0·44; KI, 0·000025. (P and K is provided in yeast and caseinogen.)

[2] Wheat, 50; barley, 15; oats, 10; yellow corn, 10; millet, 5; hemp, 5; rice, unpolished, 5.

Fig. 1

Fig. 4.

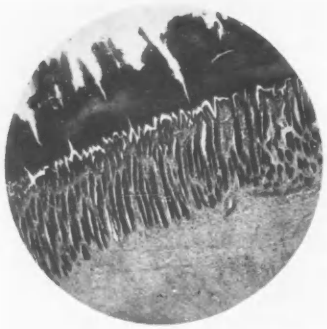


Fig. 2*a*.

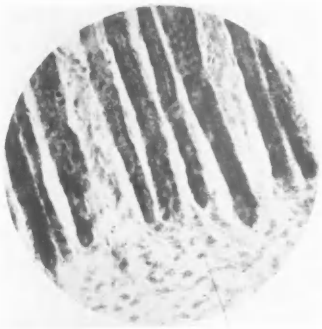


Fig. 2*b*.

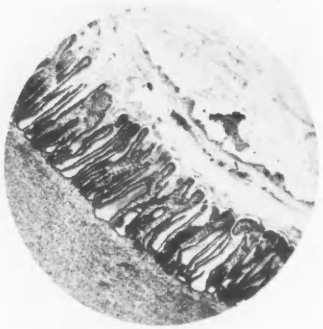


Fig. 3*a*.

Fig. 3*b*.

UNIV. OF MICH.

### Results and discussion.

The experiments clearly show that ascorbic acid does not prevent the development of the syndrome. The cause of the disease cannot be a diminished absorption of vitamin C from the intestine or a diminished production of vitamin C in the organism since subcutaneous injections of ascorbic acid were ineffective. (Owing to the close clinical resemblance to scurvy it might, however, be of interest to investigate the possibility of some relation to vitamin C in other respects.)

The disease does not seem to have any relation to the salt mixture since two quite different salt mixtures can be used in the basal diet.

The only probable explanation seems to be that the cereals of diet 66 contain a factor or factors the presence of which prevents the syndrome.

Whether the haemorrhages and the changes in the gizzard are due to the lack of one and the same factor and are necessarily connected with anaemia and insufficient growth is being investigated further.

It must be considered unlikely that the factor is vitamin A or D because of the relatively large supply of cod-liver oil.

Symptoms of polyneuritis or pellagra were never observed in connection with diets 49 and 60 (but those animals on the basal diet which reached an age of more than about 20 days showed signs of leg weakness), thus rendering it unlikely that the syndrome is due to a deficiency of vitamin $B_1$ or $B_2$.

Experiments not described in this paper in which polyneuritis and pellagra were deliberately produced did not show any acceleration or aggravation of the syndrome in question.

### Summary.

A syndrome resembling scurvy has been observed in chicks reared on an artificial diet. The symptoms are: a tendency to large haemorrhages; certain pathological changes in the mucous membrane of the gizzard; anaemia; insufficient growth.

Ascorbic acid given by mouth or subcutaneously does not prevent the disease, the cause of which is ascribed to the lack of a factor or factors occurring in cereals.

Thanks are due to Dr K. Overgaard for assistance in providing the photomicrographs.

### REFERENCES.

Dam (1929). *Biochem. Z.* **215**, 475.
—— (1930). *Biochem. Z.* **220**, 258.
Hart, Halpin and Steenbock (1920). *J. Biol. Chem.* **43**, 321.
—— Steenbock and Lepkowsky (1924). *J. Biol. Chem.* **60**, 341.
—— —— —— and Halpin (1925). *J. Biol. Chem.* **66**, 813.
McFarlane, Graham and Richardson (1931). *Biochem. J.* **25**, 358.
Plimmer and Rosedale (1923). *Biochem. J.* **17**, 772.

## EXPLANATION OF FIGURES IN PLATE VIII.

Fig. 1. Shows a pathological gizzard.
Fig. 2. Shows (*a*) the normal mucous membrane of the gizzard (the secretory lining has been somewhat frayed by the preparation); (*b*) the same more enlarged.
Fig. 3. Shows (*a*) dilated glands without haemorrhages; (*b*) the same more enlarged.
Fig. 4. Shows glandular changes with haemorrhagic infiltration of the secretory lining.

# CLXXXIV. CONDITIONS OF HYPERTROPHY OF SEMINAL VESICLES IN RATS.

## II. THE EFFECT OF DERIVATIVES OF OESTRONE (MENFORMON).

By KÁROLY DAVID, JOHN FREUD
and SAMUEL ELXEVIER DE JONGH.

*From the Pharmaco-therapeutic Laboratory of the University of Amsterdam.*

(*Received June 15th, 1934.*)

Hypertrophy of smooth muscle and narrowing of the cavity in seminal vesicles of rats following treatment with oestrone (menformon) has been previously described [for references see Freud, 1933, 1, 2]. While stimulating the growth of non-epithelial structures, oestrone damages the columnar epithelium of seminal vesicles by reducing the surface of their cavity, hastening shrinkage of cells and desquamation in castrates. The detached epithelial lining often conglomerates in masses reminiscent of tumours or embryonic tissue. Eosinophil leucocytes invade the smooth muscle and submucosa.

Male hormone (hombreol[1], assayed on combs of capons) maintains or even increases the ramification of the cavity, prevents shrinkage and desquamation of columnar cells and stimulates them to secretion and mitosis. Hombreol does not interfere with the growth-stimulating effects of oestrone on the wall of the vesicles. The two substances mutually enhance their hypertrophic actions (pace-making). Contrary to the one-sided effect of oestrone, sufficiently large doses of hombreol (5 or more c.u. daily) stimulate general hypertrophy of seminal vesicles, even when thoroughly purified from contamination with oestrogenic substances.

*Correspondence between the effects of oestrone in males and females.*

Castrated female rabbits and rats, when treated with 100–1000 m.u. of oestrone daily during several weeks, have very thick uteri, with a narrow unramified cavity. Epithelium shrinks and becomes partially detached and conglomerated. The thickened wall consists for the most part of enormously hypertrophied submucous or interstitial fibrous tissue, reminiscent of fibroid tumours (see Fig. 1). An infiltration with eosinophil leucocytes is also noticeable. This favours Witherspoon's theory [1933] concerning the possible causes of uterine fibroids in human beings. Ramification of the uterine cavity and hypertrophy, mitosis and secretion of the epithelium are stimulated by the hormone of the corpus luteum (progestin), which is thereby proved to be a female analogue of hombreol. The combined effects of oestrone and progestin on the uterus are

[1] The commercial names of the substances when here referred to are used because they are short, and also imply a certain proved freedom from unwanted contaminations. Preparations less thoroughly purified are not certain to give results comparable with those here described.

The term oestrin is used as a generic name for oestrogenic substances such as may be obtained by simple extraction of urine; individual members of this class are oestrone (ketohydroxyoestrin, menformon) and oestriol (trihydroxyoestrin).

well known. According to our experience hombreol and progestin are not interchangeable with reference to seminal vesicles and uterus, and progestin does not stimulate comb-growth in capons [de Fremery *et al.*, 1932]. Its effect on the prostate is insufficiently known.

Fig. 1. Uterus of rabbit 58 after 6 weeks' treatment with 1000 M.U. of menformon daily. (Castration 8 weeks previously.)

1. Surface.
2. Longitudinal } muscle layers.
3. Transverse }
4. Submucous connective tissue enormously thickened.
5. Cavity without epithelial lining.

Oestrone stimulates thickening and cornification of vaginal stratified epithelium. De Jongh [1933, 1, 2; 1934], after treating male castrated mice with 2–50 M.U. of oestrone daily for 20 days, found stratified epithelium replacing columnar at the orifice of the ducts of the prostate and seminal vesicle, a level at which stratified epithelium never normally occurred. The prostatic ducts were cornified and the concentric desquamated epithelium which tended to obstruct the narrow ducts. When oestrone treatment was discontinued this abnormally localised (perhaps metaplastic) tissue soon disappeared[1]. Similar observations were published by Lacassagne [1933]. De Jongh has further shown that hombreol effectively prevents metaplasia and cornification, just as a functioning corpus luteum, producing progestin, the female analogue of hombreol, is said by many workers [de Fremery *et al.*, 1932; Hisaw *et al.*, 1932] effectively to antagonise vaginal cornification. In male animals under forced treatment with oestrone the bladder is often engorged with urine so that its apex reaches the xiphisternum. Burrows and Kennaway [1934] have obtained similar results. No anatomical structure was found to obstruct the urethra, hence the retention may reasonably be assumed to be functional and similar to the "prostatisme sans prostate" of human pathology.

Male hormone promotes the development of spongy glandular structures in the male genital region and reduces the experimental incidence of engorged

[1] In rats metaplasia occurs more readily in the seminal vesicles than in the prostatic ducts.

bladder. This observation may strengthen the theoretical basis for the treatment of prostatic hypertrophy by hombreol, suggested by Prof. Laqueur and successfully applied by van Cappellen [1933, 1, 2] and others.

Finally, the growth-stimulating effect of oestrone on accessory skin-organs, such as mammary and preputial glands, is similar in males and females. A special study of the preputial glands is in progress [see also Freud, 1933, 1, 2].

*Pharmacology of oestrin.*

The compiled data show that the conception of sex-specificity of oestrin, and perhaps of "sex-hormones" in general, is untenable. In terms of pharmacology, the known "sexual hormones" act selectively on certain tissues of specialised function. In secondary sex organs oestrone stimulates growth:

(*a*) of smooth muscle tissue and interstitium;
(*b*) of submucosa and stratified epithelium;
(*c*) of submucosa and undifferentiated cylindrical epithelium;

the last, however, only in non-tubular organs, where smooth muscle is represented by interspersed fibres; in tubular organs a slight initial epithelial hypertrophy is soon superseded by that of the extra-epithelial structures of the wall, with results deleterious to the epithelium, unless hombreol (in males) or progestin (in females) restores the equilibrium between these tissues. Hombreol and progestin promote the spread, growth and differentiation of columnar and glandular epithelium. According to Pompen [1932] oestrone not only stimulates uterine growth but also sensitises the uterine muscle to functional stimuli, such as pitocin.

*Oestrin derivatives.*

While we were engaged in ascertaining these facts, new oestrogenic or chemically related substances were being discovered in various laboratories. To all these, according to present knowledge, a ring structure is ascribed similar to that of oestrone. The first of these to be tried in males was oestriol (Marrian); the experiments are described in the previous paper of this series. The oestrogenic activity of this substance and its growth-stimulating effect upon smooth muscle are very weak, but the synergism with hombreol almost equals that of oestrone.

In this paper will be described, comparatively with oestrone, the effects of the following substances on the seminal vesicles and other male accessories of immature castrated male rats:

I. *Oestrone* (crystalline menformon), extracted from the urine of pregnant mares. M.P. 255·5–256·5°; $[\alpha]_D^{20}+163°$ (in dioxan).

II. *Oestriol*, extracted from the urine of pregnant women, essentially according to the method described by Marrian [1932]. M.P. 273–276°; $[\alpha]_D+58°$ (in dioxan). Marrian has found M.P. 278–280°.

III. *Equilenin.* We are grateful to Dr Girard of Paris for supplying this substance. The constants determined in this laboratory were: M.P. 254–255°; $[\alpha]_D^{20}+88°$ (in dioxan). According to Girard [1932] himself, they were: M.P. 258–259°; $[\alpha]_D^{20}+87°$.

IV. *Equilin*, separated from crude oestrone crystals by repeated fractionation, white platelets crystallised from dilute alcoholic solution. M.P. 236–237·5°; $[\alpha]_D+307°$ (in dioxan). (Found: C, 80·64; H, 7·39 %. $C_{18}H_{20}O_2$ requires C, 80·55; H, 7·52 %.) According to Girard the constants are M.P. 238–240° (corrected); $[\alpha]_D+308°$.

V. *Reduced equilin*, prepared by reduction of equilin with sodium in alcohol. M.P. 174·5–175°; $[\alpha]_D$ +220° (in dioxan). (Found: C, 79·56; H, 8·37 %. $C_{18}H_{20}(OH)_2$ requires C, 80·0; H, 8·15 %.)

VI. *Oestradiol* (dimenformon), prepared by reduction of oestrone with sodium in alcohol. M.P. 176–177°; $[\alpha]_D$ +80·5° (in dioxan). (Found: C, 79·49; H, 8·98 %. $C_{18}H_{22}(OH)_2$ requires C, 79·36; H, 8·89 %.) From diluted alcohol the substance crystallises in shiny platelets, which variously contain either 0·5 mol. $C_2H_5OH$ or 0·5 mol. $H_2O$.

VII. *Monobenzoate of oestradiol*, prepared by benzoylation of oestradiol in *N* NaOH solution. Small white needles (from diluted alcohol). M.P. 194–195°. Schwenk and Hildebrandt [1933] find M.P. 187·5–190°. (Found: C, 79·98; H, 7·37 %. $C_{25}H_{28}O_3$ requires C, 79·74; H, 7·50 %.)

VIII. *Dibenzoate of oestradiol* is obtained by benzoylating oestradiol with benzoyl chloride in pyridine solution. White, shiny platelets or needles (from alcohol). M.P. 169·5–170·5°. (Found: C, 79·96; H, 7·13 %. $C_{32}H_{32}O_4$ requires C, 79·96; H, 6·72 %.)

IX. *Diacetate of oestradiol*, prepared by boiling oestradiol with anhydrous acetic acid. Platelets shining like mother of pearl (from diluted alcohol). These crystals exhibit electrostatic phenomena immediately after filtration through a porcelain filter. M.P. 125–126°. (Found: C, 74·18; H, 7·86 %. $C_{18}H_{22}(OCOCH_3)_2$ requires C, 74·12; H, 7·92 %.)

X. *Benzoate of oestrone* (menformon B) prepared from oestrone as under VII. M.P. 218·5–221°.

XI. *Acetate of oestrone* (menformon A), prepared from oestrone as under IX. M.P. 125·5°.

XII. *Monomethyl ether of oestradiol*, prepared by methylating oestradiol in 2*N* NaOH solution with dimethyl sulphate [Marrian, 1933]. M.P. 94–96°.

In the foregoing description certain discrepancies will be noted between the constants of the substances as determined by the respective authors and by one of ourselves (K. D.). These differences, which will be discussed in detail elsewhere, are mainly due to:

(*a*) Incomplete separation of substances, or various crystal forms of the same substance [Kofler and Hausschild, 1934] having very similar solubilities or other physical properties used in isolation.

(*b*) Insufficient number of analyses to warrant consistent results.

(*c*) Lack of agreement between substances prepared at separate places (by exchange of materials and determination of their chemical and biological data at a laboratory other than that in which the preparative work was done). Our own determinations of M.P. were done with a short-stemmed Anschütz thermometer. No corrections were applied. The optical rotations were determined by the micro-method generally used for this purpose: 2–4 mg. substance being dissolved in 0·4 ml. dioxan.

### *Animals and method.*

Immature castrated male rats were used. Transscrotal castration was done when the animals were 20–30 days old. Three days after the operation a course was begun of 10 (exceptionally 14) injections during 5 (7) days. The daily dose was given in 2 × 0·1 ml. arachis oil. The animals were killed the day after the last injection and the preputial glands (Pp.), seminal vesicles (Se.), prostate (ant. lobes) and periurethral tissue were dissected and weighed on a torsion balance. The technique of dissection was as follows.

*Preputial glands.* A triangular skin-flap is lifted, the apex being the hypochondrium, the base between the two inguines. The glands are found embedded in subcutaneous fatty tissue on the ventral surface of the abdominal wall. The ducts of the glands lead into the preputial sac and are often filled with a sticky brown secretion.

*Seminal vesicles.* The dissection of seminal vesicles is described in the previous paper of this series.

*Prostate.* The two anterior lobes are easy to isolate, when the pelvic bones are bilaterally resected laterally to the symphysis, the bladder drawn caudally until the urethra presents itself in the dorsal groove of the symphysis. The symphysis is now removed and the soft prostatic tissue cut off the urethra.

*Periurethral tissue* consists of glands not unlike the prostate, of prostatic ducts, vesicular ducts, ductus deferentes and urethra. All these structures are surrounded by a muscular funnel. The prostatic tissue adherent to the inner and outer walls and ends of this funnel is macroscopically inseparable from it. The upper limit of the funnel is at the neck of the bladder, the lower at a transverse bluish line, corresponding to strong venous vascularisation.

We have improved our experimental method over that reported in our previous paper, by working with animals of known strain, litter and data of birth. If, say, six groups of animals are used at a time, the brothers of each litter are distributed over all six groups. Comparison of the average weight can be checked by comparing the brothers individually as they fall into parallel groups. Detailed protocols cannot be reported here. Hence the results are expressed in terms of the average weight of the animals in each group on the day of killing

Table I. *Experimental results.*

| Substance injected | $\gamma$ per day | Period days | No. of rats | Average weight g. | mg. per g. rat Pp. | Pr. | Se. |
|---|---|---|---|---|---|---|---|
| None | — | — | 30 | 44 | 0·34 | 0·22 | 0·14 |
| None | — | — | 85 | 58 | 0·34 | 0·17 | 0·13 |
| I. Oestrone | 2 | 7 | 5 | 37 | 0·30 | 0·44 | 0·25 |
| | 2 | 5 | 22 | 47 | 0·39 | 0·25 | 0·18 |
| | 6 | 5 | 5 | 45 | 0·35 | 0·42 | 0·29 |
| | 10 | 5 | 5 | 39 | 0·48 | 0·39 | 0·27 |
| II. Oestriol | 2 | 7 | 5 | 44 | 0·22 | 0·28 | 0·18 |
| | 2 | 5 | 5 | 38 | 0·50 | 0·42 | 0·19 |
| | 10 | 5 | 5 | 35 | 0·38 | 0·48 | 0·19 |
| III. Equilenin | 2 | 7 | 5 | 44 | 0·24 | 0·25 | 0·15 |
| IV. Equilin | 6 | 5 | 5 | 50 | 0·50 | 0·30 | 0·27 |
| V. Reduced equilin | 6 | 5 | 5 | 46 | 0·53 | 0·31 | 0·40 |
| VI. Oestradiol | 2 | 7 | 5 | 40 | 0·35 | 0·25 | 0·46 |
| | 0·2 | 5 | 5 | 38 | 0·21 | 0·31 | 0·19 |
| | 0·3 | 5 | 5 | 33 | 0·28 | 0·30 | 0·21 |
| | 0·5 | 5 | 10 | 40 | 0·31 | 0·39 | 0·31 |
| | 1 | 5 | 5 | 42 | 0·37 | 0·34 | 0·27 |
| | 2 | 5 | 27 | 48 | 0·40 | 0·30 | 0·37 |
| | 4 | 5 | 5 | 50 | 0·42 | 0·31 | 0·32 |
| | 6 | 5 | 5 | 38 | 0·45 | 0·74 | 0·45 |
| VII. Monobenzoyloestradiol | 0·5 | 5 | 5 | 41 | 0·49 | 0·54 | 0·29 |
| | 2 | 5 | 10 | 40 | 0·45 | 0·51 | 0·45 |
| | 6 | 5 | 10 | 43 | 0·45 | 0·58 | 0·46 |
| | 10 | 5 | 5 | 48 | 0·41 | 0·38 | 0·42 |
| VIII. Dibenzoyloestradiol | 2 | 5 | 5 | 46 | 0·20 | 0·31 | 0·28 |
| | 6 | 5 | 5 | 44 | 0·40 | 0·58 | 0·22 |
| IX. Diacetyloestradiol | 0·5 | 5 | 5 | 40 | 0·37 | 0·42 | 0·15 |
| | 2 | 5 | 5 | 43 | 0·44 | 0·40 | 0·40 |
| X. Benzoyloestrone | 2 | 5 | 8 | 55 | 0·38 | 0·13 | 0·16 |
| XI. Acetyloestrone | 2 | 5 | 8 | 54 | 0·34 | 0·12 | 0·23 |
| XII. Monomethyloestradiol | 2 | 5 | 5 | 39 | 0·30 | 0·29 | 0·17 |
| | Total | | 320 | | | | |

and the weight of the various organs in mg. per g. rat. The average weight multiplied by the average mg. per g. organ weight gives the actual average organ weight in every group.

The increase in weight of the periurethral tissue as a result of treatment is mainly due to enlargement of the prostatic ducts and lateral lobes of the prostate. The periurethral tissue is not included in the table, the difference of its average weight in experimental and control groups being added to the weight of the prostate (Pr.).

## Discussion.

The *periurethral tissue* of 115 control animals averages 0·37 mg. per g. rat. The largest average actual prostatic weight of all groups was 0·32 mg. per g. rat. In those groups, where very large Pr. weights are noted in the table, this higher value is due to increase inside the periurethral muscle funnel which was added as a correction to the actual prostatic weight, as noted in the previous paragraph. Such is the case in groups I, II, VI, VII, VIII and IX, mainly where large doses were administered. De Jongh observed profound histological changes inside the funnel as a result of the prolonged administration of oestrin derivatives. These changes are in some respects analogous to human prostatic hypertrophy.

We chose to compare the effects of various substances according to the weight of the dose rather than to the oestrogenic potency expressed in units. Certain materials have practically no oestrogenic effect whatever; concerning others there is no agreement in the different laboratories; one might therefore assume that oestrogenic effects such as are described are in certain instances due to contamination.

*Preputial glands* respond to larger dosages in groups I, II?, IV, V, VI, VII and IX.

*Prostates* respond strongly in the groups already noted. Considering however that the largest average weight of the free prostate is 0·32 mg. per g., it is apparent that the growth of this organ and of the preputial glands is stimulated at most only moderately by the substances here used. Anticipating some unpublished experimental data concerning the effect of male (comb-growth) hormone on these two organs, we may state that with the same type of treatment (or one day less) the growth of prostate and preputial glands may be stimulated by male hormone considerably more than by the largest doses of any of the substances here mentioned. Seminal vesicles stand in a sharp contrast to the other organs and structures in this respect, in that:

(*a*) they remain unaffected by: equilenin (III), benzoyloestrone (X), and monomethyloestradiol (XII);

(*b*) they are moderately influenced by: oestrone (I), oestriol (II), equilin (IV), less than 0·5$\gamma$ daily of oestradiol (VI), less than 0·5$\gamma$ daily of monobenzoyloestradiol (VII) and dibenzoyloestradiol (VIII), less than 1–2$\gamma$ daily of diacetyloestradiol (IX), acetyloestrone (XI);

(*c*) they are strongly stimulated to growth by: reduced equilin (V), oestradiol (VI), its monobenzoate (VII) and diacetate (IX), in doses of or above 2$\gamma$ daily for 5 days.

The weight of the seminal vesicles after the usual treatment is increased to nearly three times that of the controls.

Within certain limits increasing doses have increasing effects. So does prolonged treatment, even if prolonged by only 2 days.

Histological comparison reveals no qualitative difference from the effects of oestrone.

Oestradiol and its active esters cause muscular hypertrophy and ultimately destroy cylindrical epithelium; their relation to the effect of male (comb-growth) hormone is the same as that which was noted in the first section of this paper.

The increased oestrogenic potency of these substances is associated with an increased effect on the seminal vesicles. Oestradiol is about twice as oestrogenic as oestrone [David, 1933], but its monobenzoate and diacetate are (*qua* oestrus) almost the same as oestrone. Substances which have no effect on the seminal vesicles are not oestrogenic. The dibenzoate of oestradiol has no oestrogenic effect administered in the usual doses (more than $5\gamma = 1$ mouse unit); its moderate influence upon seminal vesicles may be due to some contamination with the mother substance or to a very slow hydrolysis of the ester in the body; the latter is a possible explanation of the marked activity of the diacetate of oestradiol and other esters.

The purpose of this study of oestrin derivatives was to investigate certain effects of these substances apart from their oestrogenic capacity. It was thought possible that a modification of chemical structure might permit of a qualitative or quantitative differentiation of myotropic and epitheliotropic effects. Although we were able to show clearly that the muscular effect may increase relatively more than the epitheliotropic, as in the case of oestradiol, its monobenzoate and its diacetate (about 4–7 times more active upon muscle tissue, while only twice as active upon oestrus), yet on the basis of these experiments, neither of the two distinct functions could be consistently associated with the phenolic or aliphatic hydroxyl or ketone groups in the molecular formula of oestrin.

The myo- and epithelio-tropic effects may be regarded as two functions, regularly associated with the oestrogenic substances and their known derivatives. The former may be seen in the muscular walls of the uterus or of the seminal vesicles, the latter in the vaginal epithelium or in the metaplastic changes which occur in the excretory ducts of the prostate and seminal vesicles. The presence or absence of such effects, as well as of the other effects of oestrin (*e.g.* on the mammae, preputial glands, uterine vessels, feathering of birds and the antimasculine action) should be investigated for every new derivative of oestrin, to ascertain its complete pharmacological and physiological significance.

## Summary.

1. Sex hormones are not sex-specific, nor do they specifically affect whole organs but only certain tissues. In sex organs oestrone (menformon) and certain of its derivatives are growth-promoters to the smooth muscle, stratified epithelium and some glandular epithelia of ectodermal origin, the latter when not located in tubular organs with strong muscular walls, such as mammae and, to a moderate degree, the preputial glands.

2. Glandular epithelium in its development to the secretory stage is subject to either male or corpus luteum hormone; these probably also counteract noxious effects of oestrin derivatives upon the structures concerned.

3. An unsuccessful attempt was made to associate the epitheliotropic and myotropic effects of oestrogenic substances and derivatives with certain known alterations of their structure.

4. However, it was ascertained that oestradiol, its monobenzoate, its diacetate and also reduced equilin, all have myotropic effects which are higher than that of oestrone and some other substances derived from or related to oestrone.

5. Between oestrogenic and myotropic effects a rough parallelism was found, though within this parallelism a limited number of experiments suggest a comparatively enhanced myotropic effect for substances named in paragraph 4.

6. Without an extensive investigation of their actions on the various components of the primary and secondary sex glands of the male and female, our knowledge of the substances belonging to the chemical group of oestrin will necessarily remain incomplete in its most important features.

## REFERENCES.


Burrows and Kennaway (1934). *Amer. J. Cancer*, **20**, 48.
van Cappellen (1933, 1). *Ned. Tijdschr. Gen.* **77** (ii), 16.
—— (1933, 2). *Deutsch. med. Woch.* **59**, 19, 726.
David (1933). *Acta Brev. Neerl.* **3**, 160.
de Fremery, Luchs and Tausk (1932). *Pflüger's Arch.* **231**, 352.
Freud (1933, 1). *Biochem. J.* **27**, 1438.
—— (1933, 2). *Acta Brev. Neerl.* **3**, 123.
Girard (1932). *Compt. Rend. Acad. Sci.* **195**, 181.
Hisaw, Leonard and Fevold (1932). *Amer. J. Physiol.* **100**, 111.
de Jongh (1933, 1). *Ned. Tijdschr. Gen.* **77**, 3742.
—— (1933, 2). *Acta Brev. Neerl.* **3**, 112.
—— (1934). *Ned. Tijdschr. Gen.* **78**, 11.
Kofler and Hausschild (1934). *Z. physiol. Chem.* **224**, 150.
Lacassagne (1933). *Compt. Rend. Soc. Biol.* **113**, 590; **114**, 870.
Marrian (1932). *Biochem. J.* **26**, 25.
—— (1933). *Biochem. J.* **27**, 311.
Pompen (1932). Dissertation. Amsterdam.
Schwenk and Hildebrandt (1933). *Naturwiss.* **21**, 177.
Witherspoon (1933). *Endocrinology*, **17**, 703.

# CLXXXV. THE ACTION OF THYROXINE ON THE MILK AND MILK-FAT PRODUCTION OF COWS.

By WILLIAM RICHARD GRAHAM, jun.

*From the National Institute for Research in Dairying, Shinfield. nr Reading, England.*

(*Received June 15th, 1934.*)

A previous study of the effects of thyroidectomy and thyroid feeding on the lactation of cows has shown that the thyroid gland has a marked influence on the production of milk and milk-fat by the cow [Graham, 1934]. Rises in milk and milk-fat production were shown when desiccated thyroid glands were added to the diet either of thyroidectomised animals or normal animals which were past the peak of the lactation cycle. Milk-fat production appeared to be more closely related to the action of thyroid than was milk secretion. The experiments suggested some relationship between milk-fat formation and metabolic rate.

In investigating this action more fully, it was thought advisable to pin it down, if possible, to thyroxine. If thyroxine were actually shown to reproduce the results found after feeding the dried glands, there are several possible mechanisms by which its activity in this direction could be exercised. If it is an effect directly due to changes in metabolic rate, it ought to be possible to bring about a similar effect with nitrophenols.

## Experimental.

Five cows past the peak of the lactation cycle were treated as shown in Table I after a control period of 3 weeks. It will be noted that three animals

Table I. *Showing the daily amounts of various substances fed to cows during the 6 weeks experimental period.*

| Cow No. | Week of experiment | | | | | |
|---|---|---|---|---|---|---|
| | 4 | 5 | 6 | 7 | 8 | 9 |
| 1 | Thyroxine 20 mg. | Thyroxine 5 mg. | Thyroxine 5 mg. | Thyroxine 10 mg. | Thyroxine 10 mg. | Thyroxine 20 mg. |
| 2 | 2:4-Dinitro-phenol 1 mg./kg. | 2:4-Dinitro-phenol 2 mg./kg. | 2:4-Dinitro-phenol 1 mg./kg. | *Dried thyroid glands ½ lb. | *Dried thyroid glands ½ lb. | *Dried thyroid glands ½ lb. |
| 3 | *Dried thyroid glands ½ lb. | Dried thyroid glands ½ lb. | Dried thyroid glands ½ lb. | Dinitro-phenol 1 mg./kg. | Dinitro-phenol 1 mg./kg. | Dinitro-phenol 1 mg./kg. |
| 4 | †Prolactin 10 ml. | Prolactin 10 ml. | Prolactin 10 ml. | Prolactin 3 days 20 ml. | — | Thyroxine 20 mg. |
| 5 | †Prolactin 10 ml. | Prolactin 10 ml. | Prolactin 10 ml. | — | Thyroxine 10 mg. | Thyroxine 20 mg. |

* ½ lb. represents the original wet weight of the amount of dried material fed.

† 10 ml. prolactin were equivalent to 100 mg. of the active precipitate of the method of preparation (activity tested on guinea-pig).

received injections of thyroxine at some time during the experimental period. Two cows were also given small amounts of 2:4-dinitrophenol in their diets. The milk secretion of the same two animals was shown to be sensitive to the feeding of dried thyroid glands. Daily injections of 100 mg. of the active precipitate of prolactin, prepared by two methods from pituitary glands, were made in two animals. The mammary reaction of these two animals was subsequently shown to be sensitive to thyroxine.

## Results and discussion.

Fig. 1 shows the effect of injections of thyroxine on the milk and milk-fat production of cow 1. The effect of essentially similar treatment on other animals is demonstrated with cow 4 (9th week, Fig. 4) and cow 5 (8th and 9th weeks, Fig. 5). The results appear to confirm the view that the presence or absence

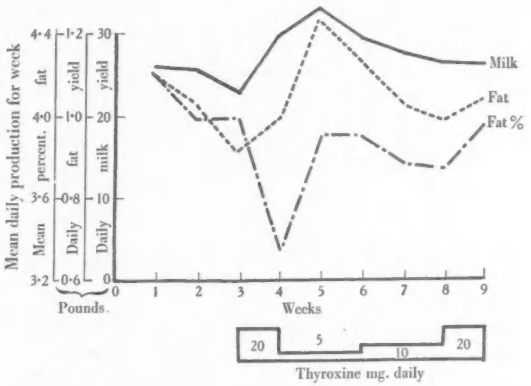



Fig. 1. Showing the effect of injections of thyroxine on the milk yield, milk-fat yield and percentage of milk-fat of cow 1.

of thyroxine itself is the essential condition responsible for the effects of thyroid feeding and thyroidectomy on milk secretion. A marked rise in milk fat production is shown in each instance after the administration of thyroxine. The effect on total milk secretion is again variable. The milk secretion of cow 1 rose from 22·9 lbs. to 32·9 lbs. for the daily average of the week when 20 mg. of thyroxine were injected daily. This represents a rise of over 40 % while the total secretion of cow 5 was hardly affected by thyroxine injections. Cow 4 showed a significant rise in milk secretion during the last week of the experiment when it was receiving thyroxine. Reduction of the quantity of injections in cow 1 was followed by decreases in both total milk and milk-fat production; only milk fat production however rose when the maximum dose was again given.

The results of feeding 2:4-dinitrophenol are shown in Figs. 2 and 3. When this substance was fed at the rate of 1 mg. per kg. of body weight (approximately 500 mg.) daily, neither milk secretion nor fat production was altered, nor was this amount in the diet able to maintain the secretion of cow 3 already stimulated by thyroid feeding. When 2 mg. per kg. were fed to cow 2 during the 5th week of the experiment fat production rose 0·03 lb. daily giving a rise of 0·18 in the butter fat percentage. This rise is of the order of 4 % and may

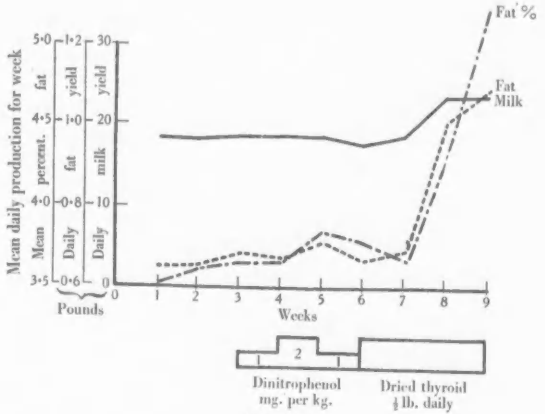



Fig. 2.

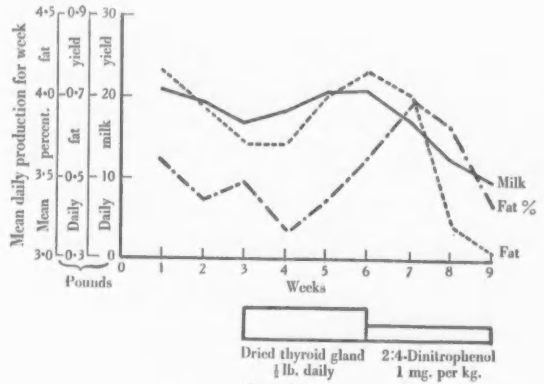



Fig. 3.

Figs. 2 and 3. Showing the effect of feeding 2:4-dinitrophenol or desiccated thyroid glands on the milk yield, milk-fat yield and milk-fat percentage of cows 2 and 3.

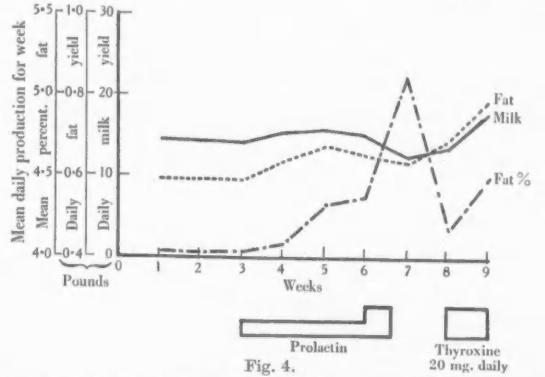



Fig. 4.

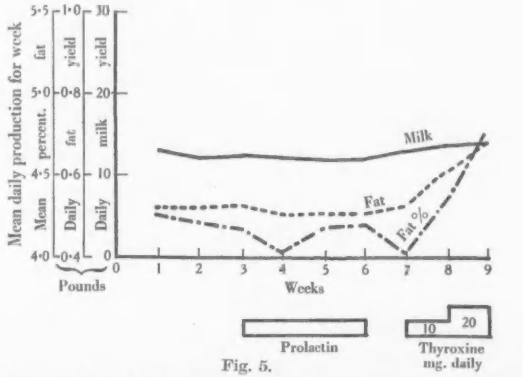



Fig. 5.

Figs. 4 and 5. Showing the effect of injections of prolactin or thyroxine on the milk yield, milk-fat yield and milk-fat percentage of cows 4 and 5.

or may not be significant. The weight of milk secreted remained constant. Dinitrophenol had been purposely used with great caution since nothing is known of its effects when fed to this species. No pyrexia was observed, however, with the amounts used.

The two preparations of prolactin were injected daily into cows 4 and 5 (Figs. 4 and 5). Cow 4 received a preparation made by the method of Lyons and Catchpole [1933] while cow 5 was given one prepared by the method of Riddle *et al.* [1933]. The injections into cow 4 were followed by a slight rise in secretion which later fell. Fat production rose appreciably and fell more slowly than milk secretion as shown by the rising fat content of the milk from the 5th week. Doubling the amount of injections for 3 days during the 6th week augmented rather than checked the fall in secretion. Cow 5 showed no change in the secretion of either milk or milk-fat after receiving daily injections of prolactin for a period of 3 weeks.

While the results given by cow 4 resemble those after the administration of thyroxine to some extent, the preparation injected was grossly contaminated with protein. The facts that the animal had developed a body temperature above the normal by the 7th week and that doubling the amount of the injections was followed by a fall in secretion make it appear that the results obtained might be due to the effect of injecting a foreign protein rather than to the uncomplicated effect of prolactin. The preparation injected into the other animal was apparently more free from protein and caused no changes in secretion.

In conclusion one may point out that each animal at one time or another during the course of the experiment came under the influence of added thyroxine either as thyroid glands in the diet or as the pure hormone. The rise in milk-fat production was always marked. The rise in milk secretion, however, showed large variations giving more evidence that the cause of the increase in total milk secretion may not be identical with the cause of the rise in milk-fat. It may or may not be a significant observation that cows with a fine texture of skin show a much smaller milk secretion-response to thyroxine than do those with coarse hides.

## Summary.

Thyroxine is the principle in the thyroid gland which causes a marked increase in the production of milk fat. Thyroxine also causes a less regular increase in milk secretion when given to cows during the period of declining lactation.

The author wishes to thank Messrs Allen and Hanbury, Ltd., for one of the preparations of prolactin used and also to thank A. Wagstaff for his technical assistance.

## REFERENCES.

Graham (1934). *J. Nutrition*, **7**, 407.
Lyons and Catchpole (1933). *Proc. Soc. Exp. Biol. Med.* **31**, 299.
Riddle, Bates and Dykshorn (1933). *Amer. J. Physiol.* **105**, 191.

Fig. 4. Chart 8. Showing the effect on injections of prolactin or thyroxine on the milk yield, milk-fat yield and milk-fat percentage of cows 4 and 5.

# CLXXXVI. PROBLEMS OF NITROGEN CATABOLISM IN INVERTEBRATES.

## I. THE SNAIL (*HELIX POMATIA*).

BY ERNEST BALDWIN[1] AND JOSEPH NEEDHAM.

*From the Biochemical Department, Cambridge.*

(*Received June 19th, 1934.*)

### I. INTRODUCTION.

IN the livers of animals having a ureotelic metabolism, as Clementi [1915; 1918] has pointed out, the enzyme arginase is present, whereas those which have a uricotelic metabolism do not possess it. This principle, though valid for members of the vertebrate phylum, has not as yet been successfully extended to the invertebrates owing to certain serious difficulties. Firstly, arginase seems to be rare in its occurrence among such animals (further work upon this distribution is now in progress); secondly, few invertebrates excrete notable quantities of urea. Clementi [1918] has examined the hepatopancreas of *Astacus fluviatilis* and *Helix pomatia* and the whole bodies of termite larvae, while Hunter and Dauphinee [1924] have further studied the caecae of a starfish (*Pisaster*), the hepatopancreas of *Cancer* and the digestive glands of a clam (*Saxidomus*). All these organs, with an exception in the case of *Helix*, proved to contain no detectable amounts of arginase. Perhaps this is not surprising, since many invertebrates rely upon argininephosphoric acid for their muscular activity [Meyerhof and Lohmann, 1928; Meyerhof, 1928; Needham *et al.*, 1932], so that high concentrations of arginase might be incompatible with the efficiency of the muscle.

For comparison with these results the findings of Delaunay [1927] are of great interest. The following figures, taken from that author, express the amounts of urea-N excreted by various invertebrates as percentages of the total nitrogenous excretion:

| | | | | |
|---|---|---|---|---|
| *Invertebrates* | AQUATIC ... | ... | Crustacea, *e.g. Maia*<br>Cephalopoda, *e.g. Sepia*<br>Echinodermata, *e.g. Echinus* | Average 7–8 % |
| | TERRESTRIAL | ... | Annelida, *e.g. Lumbricus* | 16 % |
| | | | Mollusca, *e.g. Helix* | 22 % |
| | | | *Arion* | 71 % |
| *Vertebrates* | ... ... | ... | Mammalia | 90 % |

There is thus a striking correlation between the presence of arginase in the terrestrial pulmonate gastropods and the fact that they appear to excrete a relatively large percentage of their nitrogen in the form of urea.

The enzyme arginase has long been known to hydrolyse arginine into urea and ornithine, and for many years our knowledge of its functions went no further. Birds, for example, obtain the ornithine used for their detoxication processes from arginine by means of the arginase which is present in their kidneys. Clementi [1932] has found that the whole of the small amount of urea excreted

[1] Senior 1851 Student.

by hens comes from ingested arginine, presumably by the action of arginase. But recently, thanks to the work of Krebs and Henseleit [1932], new light has been thrown on the importance of arginase [Krebs, 1934]. In the livers of mammals arginase functions, not merely as a single enzyme of high specificity performing a specific task, but as one of an organised group of enzymes by whose successive action a continuous urea production is brought about, thus:

(i) arginine → ornithine + urea;
(ii) ornithine + $CO_2$ + $NH_3$ → citrullin;
(iii) citrullin + $NH_3$ → arginine.

The system is a cyclical one and its action depends both upon the integrity of the cell structure and the concomitant processes of oxidation. The urea production of amphibian, reptilian and human foetal livers has also been studied by Manderscheid [1933] and found to follow the same lines.

It seemed to us desirable, in view of these interesting facts, to attempt to discover the origin and the mode of formation of the urea excreted by the invertebrates and to begin this study by selecting the edible snail, *Helix pomatia*, as our material. On account of its greater relative urea excretion the slug might have been preferable but we found that, unlike the snail, it is difficult to keep in the laboratory, easily suffering lethal infection by bacteria. The snail, however, is known to be highly resistant to such invasion [Gatenby and Duthie, 1932; Gatenby and Hill, 1934] and furthermore, the amount of information already available is much greater than in the case of the slug.

But several difficulties are inherent in this material. Firstly, according to Delaunay [1927; 1931] the snail excretes some 20 % of the total nitrogen in the form of urea, but this total is itself small. Hesse [1910] states that the rate of excretion corresponds to 3·85 mg. total N per kg. (presumably excluding the shell) per hour at 17·5°, which is equivalent to about 3·6 mg. total N per snail per day under our conditions. Also, the "total" value taken by Delaunay did not represent the true total, as some uric acid was neglected in his estimations, being insoluble. The total daily output of urea was therefore very small. We found that the average excretion of a fully-fed snail is about 0·4 mg. per day; the highest value found was 3·92 mg. in the case of a snail which had just woken from hibernation and eaten prior to the collection of its excreta. The basal level, reached after 5 days' starvation, is only about 0·04 mg. of urea per day. A delicate method of estimation was therefore necessary, and this was found in that of Krebs and Henseleit [1932] which we employed with no modification save that the estimations were performed at 28° instead of 37°. Some preliminary determinations gave theoretical yields.

The second difficulty was more fundamental and more troublesome. Snail tissues, according to Przyłęcki [1922], contain urease, a finding which we were able to confirm. Albrecht too [1920] found urease, and also urea, in the tissues of four marine molluscs, including one gastropod. Consequently, in attempting to find conditions under which urea formation might proceed *in vitro*, we always encountered the difficulty that urea might be broken down as well as synthesised. Fortunately no urease was demonstrably present in the hepatopancreas, as we shall see, and we were therefore able to perform many experiments with this tissue.

## II. Experimental animals and methods.

Our snails were obtained in the hibernating state from dealers. In order to waken them they were placed, epiphragm downwards, in a Petri dish containing a layer of water about 1 cm. deep. This was put in a wire cage kept inside a

special glass-fronted incubator maintained at 23–27° and 95 % humidity. Waking usually followed within 24 hours, when the snails crawled away to feed upon fresh cabbage which was kept in the cage and replaced as often as necessary.

When injection and starvation experiments were carried out the animals were kept in crystallising dishes covered by Petri dishes, the latter having previously been drilled and short glass tubes cemented over the holes. A slow stream of air could thus be passed through the dishes. A little water was placed in the bottom of each dish to keep the atmosphere moist and to aid in the collection of the excreta, the dishes themselves being kept in the incubator.

Two principal experimental methods were employed, firstly the tissue-slice technique, and secondly the injection of various substances. In the latter case, the substance to be injected was dissolved in sterile water, usually 1 ml., and injected by means of a sterile hypodermic syringe into the posterior haemocoele, passing the needle from the side of the apical end of the shell. With practice this could be accomplished with great ease, and the animals often did not even withdraw into the shell during the injection.

For tissue-slice experiments the organs were rapidly removed from the animals, care being taken to avoid contamination with slime. Adherent tissues, blood vessels *etc.* were removed and the organs gently dried on filter-paper. The slices were cut with a sharp razor blade. The hepatopancreas proved to be an exceedingly fragile tissue in the slice form, and during 3 or 4 hours' shaking about a half fragmented. The nephridium ("kidney" or organ of Bojanus [1818]), a thin walled, hollow structure, contained spherical concretions of uric acid [Strohl, 1914; Cooke, 1895]; these were removed as far as possible, and the organ was then sliced transversely. After slicing, the tissues were washed three times, for 3 minutes each time, in a special Ringer solution. After removing the excess of the washing solution, samples of the tissues were weighed out on the torsion balance; on account of the extensive fragmentation it was useless to weigh at the end of the experiment. For the same reason, in attempting to obtain a synthesis of urea *in vitro*, we did not shake the tissues in the manometer-flasks as may be done, for example, with rat-liver and kidney slices. They were instead suspended in the appropriate solutions in small conical flasks through which a special gas mixture could be passed while they were being shaken in the bath. Before taking an aliquot portion for manometric estimation, the fluid was usually filtered through glass wool. The bath was electrically maintained at 28° throughout.

Most of the work was done using a Ringer solution buffered with bicarbonate in equilibrium with the standard Warburg gas mixture (95 % $O_2$, 5 % $CO_2$). The composition of the Ringer solution followed that recommended by Bernard and Bonnet [1930]:

| | g./litre |
|---|---|
| NaCl ... ... ... ... | 5·87 |
| KCl ... ... ... ... | 0·73 |
| $CaCl_2.6H_2O$ ... ... ... | 3·96 |
| $MgCl_2,6H_2O$ ... ... ... | 5·62 |
| $NaHCO_3$ ... ... ... ... | 1·87 |
| $KHCO_3$ ... ... ... ... | 0·22 |

The freezing-point depression of this solution is $\Delta$ −0·74°. The terrestrial gastropods are exceptional in that they periodically undergo wide fluctuations in the tonicity of their internal medium; thus Arvanitaki and Cardot [1932] show that the osmotic pressure of *Helix* blood may decrease from $\Delta$ −0·47° to

$\Delta$ −0·2° after a short period of rain. Kamada, too, [1933] has demonstrated a similar difference between the body fluids of hibernating and active snails by means of vapour pressure measurements. It is therefore necessary to dilute the above Ringer-bicarbonate solution to the degree indicated by freezing-point measurements on the haemolymph of the animals in question, adding sufficient bicarbonate to make the final bicarbonate concentration $2{\cdot}5.10^{-2}M$. This concentration, suitable for mammals, seems to be not unsuitable for the snail. The gas phase in equilibrium with native *Helix* blood contains 2·9 vols. % $CO_2$ (as compared with 2·5 vols. % in the case of Warburg's mammalian solution). By the blood-gas pump method 6 vols. % $CO_2$ are found, and this is comparable with the values given in the older literature for snail blood (see *Tab. Biol. 3*, 408). By complete acidification the total $CO_2$ (including bicarbonate) is found to be roughly 45 vols. %, a value not far removed from the 55 vols. % of mammalian sera.

The following determinations showed that the above Ringer-bicarbonate should be diluted with its own volume of water.

| | $p_H$ | $\Delta$ |
|---|---|---|
| Ringer-bicarbonate | 7·5 | 0·74 |
| Snail blood (asleep) *H. pomatia* | 8·03 | 0·37 |
| ,, ,, *H. aspersa* | — | 0·40 |
| ,, (waked and fed) *H. pomatia* | 7·75 | — |
| Hepatopancreas (hibernation) | 6·87 | — |
| ,, (active) | 6·10 | — |
| Organ of Bojanus (hibernation) | 6·92 | — |
| ,, (active) | 6·60 | — |
| Gut contents (hibernation) | 5·73 | — |
| ,, (active) | 5·51 | — |

$CO_2$ was passed through to saturate the solution and the slight precipitate allowed to settle. The tissue $p_H$ was determined, following Meyerhof [1930], by grinding with sand at 0° and making electrometric measurements. It appears that waking from hibernation is associated with a slight change of the tissue $p_H$ toward the acid side.

## III. Optimum conditions.

We began our investigations by studying the conditions for the optimum survival of the tissues. From 50 to 250 mg. of the moist slices of the hepatopancreas and the nephridium were taken, and their respiration was examined at 28°. This temperature is not excessive for molluscan tissues, as Chapheau has shown [1932]. The first experiments were carried out in Ringer buffered at $p_H$ 7·4 with phosphate, manometer-cups of the Cremer type being employed and provision being made for the absorption of $CO_2$ [see Dixon, 1934]. The oxygen uptake of the hepatopancreas was linear in all cases, that of the nephridium slightly irregular. The addition of glucose stimulated the respiration considerably, that of galactose still more, while lactate had no effect:

| | % increase in $O_2$ consumption |
|---|---|
| Controls | — |
| Lactate | 6·4 |
| Glucose | 45 |
| Galactose | 79 |

In the presence of the sugars practically linear oxygen uptakes were obtained over 2–3 hours or even longer, in this case with both tissues.

We next used the indirect "improved method" of Warburg [1924] in order to compare the effects of phosphate and bicarbonate as buffering agents. Here we allowed 10–15 minutes for equilibration with the gas mixture, but after closing the taps, slight outputs of gas were observed although the gas mixture was warmed and moistened by means of coils and a bubbler immersed in the bath. The output ceased, however, after about 30 minutes and the experiments were continued for 2 hours, the oxygen uptakes being calculated over the whole period. The initial rises we attribute to glycolysis.

Our results for the hepatopancreas are summarised in Table I, which shows that bicarbonate is undoubtedly preferable to phosphate as a buffering medium.

Table I.

| | $\mu$l. $O_2$/g. wet wt./hour | | $Q_{O_2}$ | |
|---|---|---|---|---|
| Medium ($p_H$ 7·4) | Phosphate | Bicarbonate | Phosphate | Bicarbonate |
| Water | 470 | 913 | −2·35 | −4·56 |
| 1 % glucose | 682 | 900 | −3·41 | −4·50 |
| 1 % galactose | 809 | 1043 | −4·04 | −5·21 |

These $Q_{O_2}$ values are of the same order as those obtained by Chapheau [1932] in his work on another mollusc, the oyster.

It will be observed that glucose had no effect upon the respiration in bicarbonate, galactose however producing a very marked increase. This fact is of great interest in view of May's discovery [1931] that the principal polysaccharide of the hepatopancreas of *Helix* consists of "sinistrin" which, while resembling glycogen in many respects, is laevorotatory and yields only galactose on hydrolysis. Glycogen was also present, but in the eggs only galactogen was found [May, 1932].

## IV. The presence of urease.

We tested the hepatopancreas and nephridium of *Helix* for urease in two different ways. In the first place tissue-slices were shaken with a known amount of urea in Ringer buffered at $p_H$ 5 with acetate. The shaking was carried out in the cups of the manometers and a preparation of jackbean urease tipped in from a side-bulb after suitable intervals of time. Several manometers were used for each tissue and were equilibrated in the bath. The taps were then turned and one cup was tipped; the other cups at hourly intervals later. Each cup received 0·4 mg. urea, corresponding to 148 $\mu$l. $CO_2$, at the beginning of the experiment.

Two samples of the hepatopancreas evolved 140 and 145 $\mu$l. respectively, being tipped at zero time. In a third manometer a very slight uptake of gas was recorded during 2 hours' shaking, followed by the production of 141 $\mu$l. on tipping, while the fourth of the series, tipped after 4 hours, produced 141 $\mu$l. of gas. There was thus no evolution of $CO_2$ during the experiment, and a quantitative recovery of the added urea (as $CO_2$) at the end; it is therefore evident that the hepatopancreas contains no significant amount of urease.

The nephridium, however, gave very different results. One cup, tipped at zero time, gave 180 $\mu$l., 32 $\mu$l. in excess of the theoretical amount. A second cup put out 124 $\mu$l. during 2 hours' shaking, but on tipping a further 157 $\mu$l. were evolved—a total excess of 133 $\mu$l., apparently not involving the disappearance of any of the urea initially added. A third manometer was shaken for 4 hours, and this produced only 25 $\mu$l. before tipping, and 144 $\mu$l. when tipped, a perfectly quantitative recovery in this case. The interpretation of these results is difficult. Were it not for the fact that the added urea is quantitatively recovered it could

be suggested that the nephridium contains urease. As the results are so erratic it can scarcely be supposed that the pre-tipping gas production is due to preformed urea being attacked by kidney urease, since the tissue was thoroughly washed before being taken for the experiments. On attempting to repeat these results we found outputs of 0, 6 and 16 $\mu$l. before tipping, and 148, 147 and 143 $\mu$l. after tipping, the flasks being tipped after 0, 1 and 2 hours respectively.

It occurred to us that the "extra $CO_2$" could be accounted for if one supposed that urea was being produced in some way during the shaking, and broken down by a urease which was in close proximity to the synthesising enzymes but which was for some reason, *e.g.* low tissue permeability, not accessible to added urea. Yet even if this were the case, some detectable amount of the added urea would surely be attacked in 4 hours. Two further experiments threw no new light on the question. In these we took kidney slices and followed their $CO_2$ output. After an hour and a half, urea was added to one manometer and to the other a uric acid suspension obtained by carefully washing and centrifuging the milky contents of the kidney itself. In neither case was there any divergence from the previous curve. The one common factor in all these experiments was that added urea appeared not to be attacked by the kidney tissue. In view of the ease with which the jackbean enzyme can be extracted with water we decided to see whether the kidney urease could also be so extracted, for it was just possible that the thorough washing of the tissue in the previous experiments might remove the enzyme. Even so, the gas production before tipping would still be unexplained, since even glycolysis is scarcely a probable cause.

For extracting the enzyme we took, in each of two experiments, 6 nephridia and 3 hepatopancreases. Each batch of tissue was finely ground with sand and 2 cc. of Ringer solution, the mass being kept overnight in the ice-chest. Next day the solutions were centrifuged and the centrifugates treated with 1/20 volume of acetate buffer at $p_H$ 5 and again centrifuged after standing for 1 hour. Samples were now taken in the side-bulbs of the manometric flasks and added, after equilibration, to varying quantities of urea. After 7–8 hours' shaking the following volume changes had taken place in the different flasks:

| | Hepatopancreas $\mu$l. | Nephridium $\mu$l. |
|---|---|---|
| Exp. 1 | −0·7 | +14·5 |
| | −2·9 | + 6·5 |
| | −2·8 | +12·2 |
| | — | + 6·5 |
| | — | + 7·4 |
| Exp. 2 | +8·8 | +17·3 |
| | +0·7 | +13·3 |
| | +0·0 | +15·0 |
| | — | + 7·4 |
| | — | + 9·0 |
| Averages | +0·5 | +10·9 |

The figures for the hepatopancreas, together with the previous results, show that urease is entirely absent from that organ. The nephridium, however, undoubtedly contains urease [see also Przyłęcki, 1922], but the amount is certainly very small. One whole nephridium, it appears, is capable of breaking down about 0·1 mg of urea in 24 hours, or, if we suppose that only 10 % of the tissue enzyme can be extracted by our method, 1 mg. in 24 hours. This being so it is not surprising that we failed to detect it in the earlier experiments. But, small though this amount may be, the daily rate of urea output by the intact snail is of the same order.

## V. The source of the excreted urea.

The following sources suggest themselves:

(*a*) Exogenous urea.
(*b*) Exogenous arginine.
(*c*) Synthetic urea: (i) ornithine cycle; (ii) some other mechanism.
(*d*) Urea from uricolysis.

The evidence for each of these will now be considered, first that from *in vitro* experiments and then that from the injections. On account of the slow rate of urea formation in the intact snail, long-continued shaking was necessary in the slice experiments, but the risk of bacterial infection was lessened by the immunity referred to above.

### (i) *Experiments* in vitro.

(*a*) *Exogenous urea.*

Fosse [1912; 1913; 1916] has detected small quantities of urea in plant tissues; thus germinating peas yielded 640 mg. of urea per kg., while urea was also obtained from cabbage and from endives, 10 kg. of the latter being necessary for the demonstration. Unfortunately, no figures were given for the urea content of cabbage, but it must probably be very small. We ourselves prepared an extract from 5·6 g. cabbage by maceration with sand and water, boiling, filtering and evaporating. Urea was then estimated in aliquot portions and a value of approximately 1 mg. per 100 g. wet weight of cabbage was found. Now according to Stahl [1889] an average specimen of *H. pomatia* eats about 1·5 g. of cabbage daily, corresponding to 0·015 mg. urea, whereas a fully-fed snail excretes at least 0·4 mg. urea daily. Hence the ingested urea is only about one-fortieth of that excreted.

(*b*) *Exogenous arginine.*

We have already noticed that the presence of arginase has been demonstrated in the hepatopancreas of *Helix* by Clementi [1918]. We were able to confirm this and to show, moreover, that the enzyme is also present in the nephridium. The method employed was firstly to shake slices of the organs for $2\frac{1}{2}$ hours with arginine in Ringer-bicarbonate at $p_H$ 7·4 containing galactose at 28° and then after removal of the tissue and bringing the fluid to $p_H$ 5 to determine the urea content of an aliquot part; secondly to prepare a "brei" of the organ and to incubate it with arginine in phosphate buffer of $p_H$ 8·5 at 28°, afterwards proceeding as before. In both cases it was found that a considerable amount of urea was formed by hepatopancreas and nephridium. The activities, calculated in the "Arginaseeinheiten" of Edlbacher [1925; 1929], as modified by Krebs and Henseleit [1932], were as follows:

| | | Arginase units $\mu$l. urea $CO_2$ made/ mg. dry wt. tiss. × hrs. |
|---|---|---|
| Present work: | | |
| "Brei" at 28°: | hepatopancreas | 20·2 |
| Slices at 28°: | hepatopancreas | 1·3 |
| | nephridium | 7·1 |
| Edlbacher: | | |
| "Brei" at 37°: | bird-liver | 3 |
| | bird-kidney | 36 |
| | mammalian liver | 4000 |
| | mammalian kidney | 40 |
| | mammalian spleen, pancreas, heart *etc.* | 0 |

The values for the snail are therefore of the same order as those for avian tissues and for mammalian organs other than liver. The general level at which we had to work may be gauged from the fact that the average arginase content of mammalian liver is about two thousand times that of gastropod tissues, even when the latter are studied in the form of a "brei" near the optimum $p_H$ of the enzyme. For use in arguments about the state of affairs in the intact snail, the results with tissue-slices are preferable. That the arginase activity found is more than sufficient to account for the urea produced by the snail in normal life is shown by the following calculation. The maximum urea formation from the arginine added to the slices would have given 1410$\mu$l., that actually observed gave 550$\mu$l., while the expected yield, assuming that the urea excreted by the intact snail was derived solely from arginine under the influence of a uniformly distributed arginase, would have been 4$\mu$l. Thus even if the enzyme were confined to the hepatopancreas and nephridium, it would still be able to account for the normal urea output of the body, since the former organ alone could produce in one day 14 mg. urea instead of the 0·4 mg. actually observed. This conclusion, however, involves at least three assumptions, (i) that the amount of substrate is not a limiting factor, (ii) that the mechanisms for supplying the substrate to the enzyme are not inefficient, and (iii) that the activity of the urease is not considerable.

Having thus satisfied ourselves of the presence of arginase in the snail tissues we were in a position to compare the ingestion of arginine with the excretion of urea. The latter was found by placing fully-fed snails in the cages already described for 24 hours. The excreta were collected and evaporated, urea being then estimated in aliquot portions. As the $p_H$ of the water containing the excreta was ordinarily about 8·0, no destruction of urea can have taken place during the evaporation, while controls showed that not more than 10 % of the urea was destroyed by bacterial action during 24 hours. The results varied from 0·1 to 1·8 mg. per snail per day, the average figure from 22 snails being 0·4 mg. per snail per day. Now we have seen that, on the average, a snail eats 1·5 g. cabbage per day, with a protein content of 4 % of the wet weight [König, 1903]. Vegetable proteins contain on the average some 14 % of arginine [Osborne, 1924], and hence the daily intake of arginine must be at least 8·5 mg. per snail, which is equivalent to about 2·4 mg. urea, *i.e.* about six times the amount excreted.

It thus appears that the ingested arginine could amply account for the urea excreted if the urease activity of the snail is set aside. Przyłęcki [1922] found that at 37° 100 g. of snail tissue ("whole snail") can break down 76·8 mg. urea in 24 hours. The average weight of an individual is 25 g. [Künkel, 1916] so that at 28° the urease could break down 9·6 mg. in 24 hours. If we suppose that the urease normally works at its full capacity, the amount of urea produced daily must be equal to the amount excreted *plus* that broken down, *viz.* 0·4 + 9·6 = 10 mg. for the production of which 30 mg. of arginine would be required; less than one-third of the urea formed could now be accounted for by ingested arginine.

The position is therefore difficult. Of the activity and the efficiency of the urease *in vivo* we know nothing, and all calculations have to be based upon very rough average figures. It is possible to say, if we accept Przyłęcki's figures, that the limiting values for urea production are about 10 mg. per day (urease inactive) and 0·4 mg. per day (urease fully active), while the ingested arginine can account for about 2·5 mg. per day.

Thus the possibility of a synthesis cannot be said to be excluded. But the

hypothesis that arginine is the sole precursor of the urea formed gains some weight from a consideration of the magnitudes involved. The following data for the hen are calculated from those given by Clementi [1932], who clearly showed the correctness of this hypothesis in the case of the bird.

| | Weight g. | mg. urea excreted per day: Total, Fed | Total, Starving | Per kg., Fed | Per kg., Starving |
|---|---|---|---|---|---|
| Hen | 2500 | 60 | 15 | 24 | 6 |
| Snail | 20 | 0·4 | 0·04 | 20 | 2 |

It will be seen that our figures for the snail are, weight for weight, of the same order. And as we have seen, the arginase activity of snail tissues closely resembles that of the birds.

If, however, we accept the view that ingested arginine is the sole source of urea in *Helix*, we are bound to bear in mind the case of the slug, *Arion*, where the urea amounts, according to Delaunay's figures, to 71 % of the total nitrogen excreted. We feel, however, that much uric acid may possibly have escaped Delaunay's analyses (see p. 1373), and that a re-examination of the nitrogen-partition of the excreta of terrestrial Stylommatophora other than *Helix* is demanded. It is of interest here that even in mammalian tissues arginine may be the sole precursor of urea in some conditions, *e.g.* autolysis (placenta: Salaskin *et al.* [1932]; liver: Solovjev and Mardarschev [1932]).

(*c*) *Synthetic urea.*

We next proceeded to investigate the possibility of a synthesis of urea from ammonia, whether by the ornithine cycle or by some other process. The technique consisted in shaking the organ slices, in the manner described above, with bicarbonate-Ringer to which ammonia (as $NH_4Cl$) and galactose had been added, aliquot portions of the final fluid being taken for urea estimations.

Neither the hepatopancreas nor the nephridium produced any urea under the conditions employed (which are summarised in Table II) nor was any urea

Table II.

| Tissue | mg. wet weight | ml. Ringer bicarbonate | ml. 10 % galactose | $NH_3$ added mg./100 ml. | Ornithine added mg./100 ml. | Time hours |
|---|---|---|---|---|---|---|
| Hepatopancreas | 698 | 10 | 0·2 | 15·9 | — | 3 |
| Hepatopancreas | 594 | 5·0 | 0·1 | 31·8 | — | 4 |
| Nephridium | 328 | 2·5 | 0·05 | 31·8 | — | 4 |
| Hepatopancreas* | 264 | 2·5 | 0·05 | 31·8 | — | 4 |
| Hepatopancreas / Nephridium | 582 / 111 | 2·5 | 0·05 | 31·8 | — | 4 |
| Hepatopancreas | 743 | 5·0 | 0·1 | 31·8 | 20·0 | 4 |
| Nephridium | 140 | 2·5 | 0·05 | 31·8 | 20·0 | 4 |
| Hepatopancreas / Nephridium | 448 / 111 | 2·5 | 0·05 | 31·8 | 20·0 | 4 |
| Hepatopancreas† | 1630 | 4·0 | 0·08 | 31·8 | 20·0 | 3½ |
| Nephridium† | 384 | 2·5 | 0·05 | 31·8 | 20·0 | 3½ |
| Heart† | 51 | 1·0 | 0·02 | 31·8 | 20·0 | 3½ |
| Hepatopancreas | 1360 | 4·0 | 0·08 | 31·8 | 20·0 | 3½ |
| Nephridium | 485 | 2·5 | 0·05 | 31·8 | 20·0 | 3½ |
| Heart | 37 | 1·0 | 0·02 | 31·8 | 20·0 | 3½ |
| Foot muscle | 1061 | 4·0 | 0·08 | 31·8 | 20·0 | 4 |
| Gut wall | 1053 | 2·5 | 0·05 | 31·8 | 20·0 | 4 |

* *Helix aspersa*—otherwise *H. pomatia* was always used.
† Organs taken from hibernating snails.

formed when the nephridium and the hepatopancreas were allowed to co-operate. The addition of ornithine had no effect.

$Q_H$ ($\mu$l. urea $CO_2$ per mg. dry weight of tissue per hour) is here never greater than $10^{-2}$; this is within the limits of experimental error and compares strikingly with the values of 1–15 found by Krebs and Henseleit [1932] on mammalian liver. Yet the $Q_{O_2}$ of the gastropod "liver," about $-5$ at 28°, is quite comparable with that of mammalian liver, $-6$ to $-14$ at 37° [see Krebs, 1933].

In no case was any significant positive pressure set up in the manometer as a result of tipping in the urease. But all these experiments assumed some localisation of urea production. The rate of urea production in the intact snail is very small, as we have seen, and if urea synthesis were carried on more or less uniformly throughout the animal, then the magnitude of the effect to be expected might be within the limits of experimental error. We therefore performed another experiment in which the hepatopancreas was considered as an aliquot part of the whole body.

6·2 g. of liver slices were prepared from 10 snails and shaken with bicarbonate-Ringer, 0·2 ml. 10 % glucose, 0·5 ml. 1 % $NH_4Cl$ and 0·08 ml. 10 % ornithine, for 6 hours at 28° under the usual conditions. The tissue remaining intact was removed and the fluid deproteinised, boiled, filtered and evaporated to 2·06 ml., the whole of which was taken for a urea estimation. In this case the maximum possible urea formation from the added ammonia was 1065 $\mu$l., and that expected if the hepatopancreas behaved as an aliquot part of the body, 15–60 $\mu$l. Only 2–3 $\mu$l. were found, a volume within the limits of experimental error. Contributory evidence confirming this was obtained in subsequent experiments.

Hence we can only conclude that the hepatopancreas is certainly neither the main seat, nor a seat, of synthetic urea formation. Nor is urea formation confined to the gut wall, heart or foot muscle. To what extent the nephridium can be held responsible it is difficult to say on account of the fact that it contains urease. Definite evidence might be obtainable by using a specific inhibitor for that enzyme and repeating in its presence experiments upon the foregoing lines, but up to the present there is no evidence for the synthesis of urea from ammonia in any of the organs examined.

(*d*) *Uricolysis.*

The existence of enzyme systems capable of hydrolysing uric acid to give urea has been demonstrated in a number of animals by Przyłęcki [1925], Fosse *et al.* [1930] and Stransky [1933], and we have investigated the possibility that a similar mechanism might be present in the snail. The slice technique was again employed, and experiments were performed in bicarbonate buffer as well as in 0·05 % NaOH as used by Stransky. The conditions are summarised in Table III, and none of the experiments gave any evidence of urea formation.

Table III.

| Tissue | mg. wet weight | Buffer | $p_H$ | Time hours |
|---|---|---|---|---|
| Hepatopancreas | 2078 | $HCO_3'$ | 7·4 | 3 |
| Gut wall | 1122 | $HCO_3'$ | 7·4 | 3 |
| Hepatopancreas | 2910 | 0·05 % NaOH | 9·5 | 4 |
| Nephridium | 605 | 0·05 % NaOH | 9·5 | 4 |
| Gut wall | 1561 | 0·05 % NaOH | 9·5 | 4 |

Although with an acetone powder, prepared from frog-liver according to the directions of Stransky [1933], we obtained yields of only up to 15 % urea from

added uric acid under comparable conditions, we calculated that if the uricolytic activity of the snail tissues were comparable with that of frog-liver powder, the resulting urea production should have been easily detectable.

The finding by Wolf [1933] of traces of allantoin in the nephridium of *Helix* at all times of the year might be taken to indicate the presence of uricase in the animal, were it not for the fact that allantoin has been shown to exist almost universally in plants [Fosse and co-workers, 1929–34].

It is of interest in this connection that Albrecht [1920] found uricase to be absent from the alimentary tract of all the four molluscs examined by him. The enzyme has been reported as existing in the hepatopancreas of *Aplysia* by Sulima [1914] but his evidence only consisted of slight decreases in the uric acid content of tissue "brei" after 8 hours' incubation.

(ii) *Experiments* in vivo.

The foregoing experiments led to the discovery of no substance other than arginine as the precursor of the excreted urea. The following experiments were designed to supplement and extend those carried out *in vitro*, since it was possible that the conditions employed in the latter experiments were not suitable for demonstrating a synthesis of urea.

To commence this work we took four pairs of snails and starved them, urea estimations being performed each day upon their excreta. Fig. 1 shows the rate of excretion expressed as mg. urea per snail per day, plotted against the period of starvation. It will be seen that after 6 days a basal level of about 0·04 mg. per snail per day is reached, and similar results were obtained as often as the experiment was repeated.

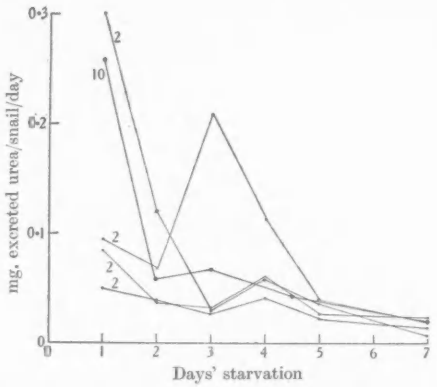



Fig. 1. The numbers on the curves refer to the number of snails used in the experiments.

It was originally our intention to feed the starving snails with filter-paper impregnated with a mixture of amino-acids, since the animals will readily ingest a great variety of substances, including even plasticine [Clements *et al.*, 1933]. We therefore made up a mixture of glycine, alanine, leucine, tyrosine, phenylalanine, tryptophan and glutamic and aspartic acids, arginine however being omitted. Unfortunately the animals refused to touch this food and we had therefore to use the injection technique already described. We had hoped to find, in the

proposed feeding experiments, that the starvation level of excretion would not be exceeded unless arginine were added to the diet; in the injection experiments, therefore, we decided to inject small doses of as many amino-acids as were sufficiently soluble and to compare the subsequent urea output with that following the administration of comparable amounts of arginine. We also tried the effect of giving various amounts of urea, with interesting results.

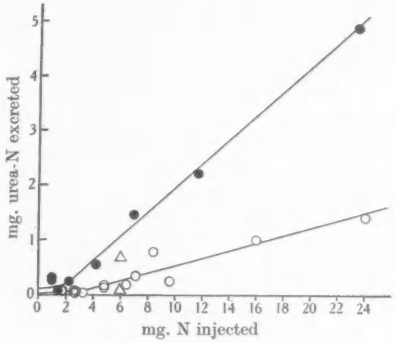



Fig. 2.

• Urea. ○ Arginine. △ Glycine.

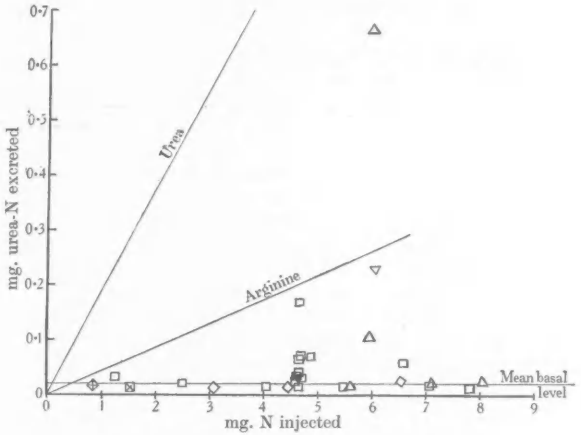



Fig. 3.

□ Alanine. △ Glycine. ◇ Valine. ⊠ Glutamic acid.
⊕ Aspartic acid. ▽ Tryptophan.

The snails were injected and kept individually or in groups of two or three under the conditions previously described and the excreta collected and worked up. As a rule, no further excess urea was excreted after the first 24 hours. Our data are given in Figs. 2 and 3, and are summarised in Table IV.

Table IV.

| Compound injected | No. of exps. done | Total mg. N injected in all exps. =N | Total mg. N excreted in all exps. =E | Basal value mg. N =B | $\frac{E-B}{N} \cdot 100$ |
|---|---|---|---|---|---|
| Arginine | 14 | 96·4 | 4·45 | 0·28 | 4·3 |
| Urea | 10 | 192 | 52·2 | 0·20 | 27 |
| Alanine | 16 | 77·0 | 0·67 | 0·32 | 0·45 |
| Glycine | 5 | 32·6 | 0·86 | 0·10 | 2·3 |
| Valine | 3 | 14·6 | 0·07 | 0·06 | 0·07 |
| Glutamic acid | 1 | 1·62 | 0·014 | 0·02 | 0·0 |
| Aspartic acid | 1 | 0·84 | 0·018 | 0·02 | 0·0 |
| Tryptophan | 1 | 6·04 | 0·224 | 0·02 | 3·3 |

It will be seen that when due allowance has been made for a basal excretion of 0·02 mg. urea-N per snail per day, 4 % of the arginine-N injected is excreted as urea. That some arginine either leaked out or was excreted unchanged was shown by the fact that a positive Weber-Sakaguchi reaction [Weber, 1930] was obtainable on a deproteinised sample of the excreta. Of urea injected, 27 % was recovered as such. The fate of the rest we have not elucidated; some urea, presumably, must have been broken up by the tissue-urease, and on the basis of Przyłęcki's data we were able to calculate that from 5–6 mg. could be so decomposed under our conditions, and more than this would probably be broken up in the intact snail. In Exp. 13, 15 mg. of urea were injected, and 3 mg. were recovered in the first 24 hours, and it is not unreasonable to suppose that the rest may have been broken down by the animal. Przyłęcki's figures were obtained with a "brei" whereas our "enzyme preparation" consisted of the intact animal. Now Przyłęcki has suggested that urease is used by the snail for controlling the $p_H$ of the blood, urine *etc.*, but we have found that doses of 25 mg. of urea already begin to show a definitely toxic effect; greater doses invariably resulted in the death of the animal. We have just seen that, by excretion and by enzymic hydrolysis, the snail can probably dispose of about 15 mg. of urea. It seems possible to us that the urease acts as a kind of detoxication mechanism, preventing the accumulation of urea in the animal. This suggestion is supported by a consideration of the tolerance towards substances other than urea.

About 20 mg. of arginine may be given and above this a toxic effect is noticed, whereas 50 mg. alanine, 38 mg. glycine and 55 mg. valine were administered without ill effect. One experiment, in which 44 mg. tryptophan were given, led to a curious result, for on the following day the entire contents of the nephridium had been extruded as though this amino-acid had a violently "diuretic" effect. The animal itself was perfectly well, however, and no trace of unchanged tryptophan could be found in the excreta.

Broadly considered, the results of Table IV show that arginine alone among the amino-acids leads to any considerable increase in the output of urea—about 4 % of the injected N. The case of glycine, where 2 % of the N administered was excreted as urea, owes its apparent importance to a single experiment (Exp. 40) as Fig. 3 shows, where 10 % of the N injected was eliminated as urea. Valine and glutamic and aspartic acids led to no excess urea excretion whatever. As regards tryptophan, only one experiment was performed; this compound has a violent diuretic effect, and much of the urea excreted could have come from the blood. According to Delaunay [1931] waking snail blood contains about 0·025 mg. urea N per ml., and one specimen of *Helix* contains some 4 ml. of blood [Cardot and Troussier, 1929] while the tissues generally contain more urea [Wolf, 1933; Delaunay, 1931], so that the urea excreted in this case,

0·2 mg., could be amply accounted for. Alanine evoked a slight excess excretion several times, but in general no urea seems to be formed from it.

But turning to examine individual cases (Fig. 3) we see that the mean basal level, about 0·02 mg. urea-N per snail per day, is frequently exceeded. This is, of course, to be expected on account of individual variation, but sometimes the basal level is exceeded to a very marked extent, *e.g.* in Exp. 35 where 30 mg. alanine led to the excretion of 0·167 mg. urea-N, a recovery of 3·6 %, which approaches the mean figure of 4·3 % found for arginine. Other high results will be seen in Fig. 3. In such cases as these, where the basal level is exceeded by as much as ten times, it is difficult to attribute the results to individual idiosyncrasy, and, indeed, these increases were absolutely erratic and not repeatable with any given snail.

It was observed that the exceptional rises were always greater when the injection was the first to which the animals had been subjected, and it occurred to us that perhaps carbohydrate might play an important part in any supposed synthesis of urea, as Krebs and Henseleit [1932] have shown to be the case in the synthesis performed by the mammalian liver. If this were so, progressive starvation might lead to diminution of carbohydrate reserves and correspondingly less urea production. The general failure to produce any urea from injected amino-acids would also be explicable on this hypothesis.

To test this we took 7 snails, previously starved till the basal rate of excretion was reached, gave these injections of ornithine, each snail receiving 36·7 mg. (=5 mg. N) and examined their excreta individually. Fig. 4 shows

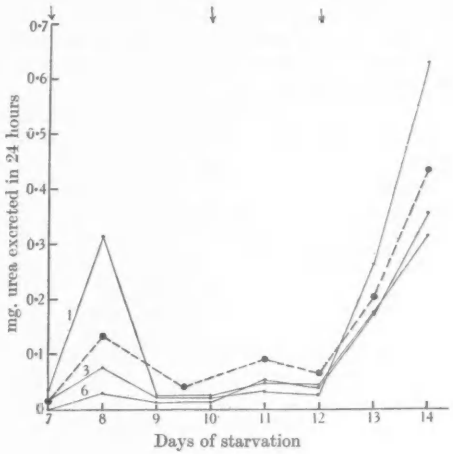



Fig. 4.

the resulting curves for the snails which survived throughout, the broken line representing the average of all the data. Two injections of ornithine were given, the excess urea output being less for the second than for the first. After this, only three snails were still alive, and these received 50 mg. glucose in addition to the usual ornithine injection. A great increase resulted and persisted for a

second 24 hours. Now whether ornithine functions here by virtue of its potential relationship with arginine, or simply as an amino-acid was not determinable by these experiments, but they suggested a new possibility. Urea might be synthesised, not from ammonia as such, but from the amino-group of the amino-acid, by a synthetic system immediately contiguous with the deaminating system, and we therefore attempted to obtain such a synthesis *in vitro*.

Hepatopancreas slices were shaken with bicarbonate-Ringer, glucose, ornithine and alanine, urea being estimated in the final fluid after removal of the tissue. The following quantities were used:

| No. | mg. tissue | Time hours | ml. Ringer | ml. 10 % glucose | ml. 10 % ornithine | ml. alanine (16·4 mg./ml.) |
|---|---|---|---|---|---|---|
| 1 | 3310 | 6 | 2·0 | 0·1 | 0·04 | 0·25 |
| 2 | 6380 | 7 | 3·0 | 0·15 | 0·04 | 0·3 |
| 3 | 7600 | $6\frac{3}{4}$ | 3·0 | 0·15 | 0·06 | 0·3 |

In the first of these experiments we obtained a total $CO_2$ output of 38 $\mu$l. but the subsequent experiments did not confirm even this small value.

Since no positive evidence seemed to be obtainable from this source we turned again to *in vivo* experiments, attempting now to decide whether or not ornithine was involved as a catalyst in the urea production previously noticed. Starving snails each received 32 mg. alanine and 10 mg. glucose, while one-half of the animals received 5 mg. ornithine in addition. Those receiving the ornithine yielded more urea than the controls and so, when the effect of this injection had passed off, the controls received the alanine *plus* glucose *plus* ornithine, the former experimental animals now becoming the controls. There was no corresponding change over in the urea excretion. A second set of injections, in which 10 mg. glucose instead of 5 mg. were given, gave exactly the same results; whichever set of snails gave the higher results in the beginning again gave high results with subsequent injections.

It is noteworthy, however, that from a given group of snails receiving alanine a greater response was obtained when carbohydrate was given than when the amino-acid was given alone.

We have therefore to conclude that, although the ingested arginine is usually sufficient to account for the urea excreted, there is also a possibility that some urea may arise from the amino-nitrogen of ingested amino-acids, without the intervention of the ornithine cycle.

## VI. The source of uric acid.

Although added ammonia leads to no urea formation we have found that uric acid is produced when ammonia is added to hepatopancreas slices shaken in bicarbonate-Ringer under our usual conditions. It was impossible to perform similar experiments upon the nephridium on account of the large amount of intracellular uric acid which is present in it, but the hepatopancreas, after thorough washing, contained only a small amount. Final estimations were made by the colorimetric method of Benedict and Franke [1922], using a Leitz compensating colorimeter, since the samples, after deproteination with acetic acid, gave green solutions with Benedict's reagent. In some experiments another difficulty was encountered in that the colours tended to fade seriously in the unknown solutions. We did not succeed in preventing this and were obliged to put the solutions into the colorimeter-cups as soon as possible after mixing.

The following data summarise the results:

| Exp. | g. hepatopancreas | Time hours | mg. uric acid expected from ammonia added | mg. found | Percentage yield |
|---|---|---|---|---|---|
| 1 | 0·6 (hibernation) | 4 | 1·98 | 1·9 | 96 |
| 2 | 4·5 (hibernation) | 6 | 3·5 | 2·0 | 57 |
| 3 | 3·36 (active and feeding) | 7 | 3·5 | 1·2 | 34 |

. It may be concluded that an almost quantitative formation of uric acid from ammonia may take place. But this cannot always be demonstrated; thus in another experiment, hepatopancreas slices incubated 6 hours with 31·8 mg. per 100 g. of ammonia gave 0·197 mg. per g. of uric acid, as opposed to 0·317 mg. produced by slices to which no ammonia had been added. It is probable that the hepatopancreas of starving animals (as in this case) is highly susceptible to the toxic action of ammonia, as has been found for the liver of the starving hen by Benzinger and Krebs [1933].

The quantity of uric acid in the nephridium is striking. At the end of hibernation the average content of a number of the kidneys was 32 mg. uric acid each, and as the average weight of a nephridium is 240 mg. the organ contains 131 mg. per g. wet weight and 660 mg. per g. dry. In other series of experiments we obtained values of 720 and 810 mg. per g. dry weight, so that it may be said that approximately three-quarters of the dry weight of the organ is uric acid. A brilliant murexide reaction is readily obtained from the nephridial contents. Marchal [1889] got 7 mg. uric acid from the nephridium or organ of Bojanus, but as he isolated it for weighing, his losses must have been considerable. Indeed, all the earlier knowledge of the nitrogen excretion of terrestrial gastropods, beginning with the work of Jacobson [1820, 1, 2; see Strohl, 1914; Wolf, 1933] could have led to the belief that they were primarily uricotelic. Unfortunately the experiments of the last-named author, which purport to bring the proof of this, do not carry much conviction. The amounts of the substances which he added to the hepatopancreas *in vitro* were out of all proportion to the amounts of uric acid formed. Thus 500 mg. urea with 5 g. hepatopancreas gave an increase of only 1·16 mg. uric acid in a typical experiment of 3½ hours' duration. The yield was never more than 1 %. As the hepatopancreas was in the form of a "brei" saturated with chloroform, which Krebs and Henseleit [1932] have shown to possess very restricted physiological properties, and as the samples of untreated "brei" show a variation of uric acid content of about the same order as the assumed new formations, it is difficult to escape the conclusion that the increases may be well within the experimental error. Quite similar remarks apply to the attempt by Sulima [1914] to demonstrate the synthesis of uric acid in the hepatopancreas "brei" of the opisthobranchiate, *Aplysia*.

We have attempted to penetrate further into the mechanism of uric acid formation in the snail by making use of pigeon tissues. It has recently been independently demonstrated by Schuler and Reindel [1933] and by Benzinger and Krebs [1933] that in the Columbidae there is a dissociation of the function of uric acid formation in such a way as to render necessary the collaboration of two organs, the liver and the kidney. Although the liver of the pigeon is unable to make uric acid itself from ammonia, it liberates into the blood-stream or into physiological salt solution a precursor-substance, which is converted by the kidney into uric acid. The fundamental experiment upon which this conclusion is based is that liver and kidney slices together, or kidney slices shaken in Ringer previously occupied by liver slices, produce much more (3 or 4

times) uric acid than either alone. As the xanthine oxidase content of the two tissues is quite similar, the phenomenon cannot be accounted for by the hypothesis of purine oxidation, for the uric acid formed should then be the sum of the two quotas formed in isolation. We have therefore attempted to ascertain whether the hepatopancreas of the snail contains any precursor substance which the pigeon-kidney can turn into uric acid, or conversely, whether the precursor known to be present in the pigeon-liver can be turned into uric acid by the snail-hepatopancreas, account being taken of purine oxidations. In this way it was hoped to discover whether the mechanism of uric acid formation is or is not identical in the bird and the gastropod. Schuler and Reindel [1933] have shown that the reaction which produces the precursor in the pigeon-liver is still functional in Willstätter acetone-ether powder preparations. We therefore prepared powders from pigeon-liver and from snail-hepatopancreas in the manner described by them.

For deproteinising the experimental mixtures after their period in the bath at 28° or 37° Rona-Kleinmann's 1·55 % uranyl acetate was used, and the uric acid was estimated by the method of Folin [1930; 1933]. The results are shown in Table V.

Table V.

All values in mg. uric acid per g. tissue or powder.

| Exp. no. | Description | Initial | Hours shaking | Final | Difference |
|---|---|---|---|---|---|
| 1 | Snail-hepatopancreas powder alone | 1·04 | 2 | 0·94 | −0·1 |
| | Pigeon-kidney slices alone | — | 3 | 0·353<br>0·391 | — |
| | Pigeon-kidney slices in the centrifugate of snail powder and Ringer | — | 3 | — | +0·32 (Pg)<br>+1·37 (Sn) |
| 2 | Snail-hepatopancreas powder alone | 1·63<br>1·48<br>1·68 | — | — | — |
| | Snail-hepatopancreas powder *plus* milk xanthine oxidase | — | 2 | 3·28 | +1·69 |
| | Pigeon-kidney slices alone | — | 3 | 0·536 | — |
| | Pigeon-kidney slices in the centrifugate of snail powder and Ringer | — | 3 | — | +1·13 (Pg)<br>+1·00 (Sn) |
| 3 | Pigeon-kidney slices alone | — | 3 | 0·346 | — |
| | Do. *plus* hypoxanthine (enough to make 3·15 mg. uric acid) | — | 3 | 2·23 | +1·884 |
| | Do. *plus* xanthine (enough to make 3·15 mg. uric acid) | — | 3 | 2·13 | +1·784 |
| 4 | Pigeon-liver powder alone | 0·156 | — | — | — |
| | Snail-hepatopancreas slices alone | 0·091 | 4 | 0·147 | +0·056 |
| | Snail-hepatopancreas slices in the centrifugate of pigeon powder and Ringer | — | 4 | — | +0·065 (Pg)<br>+0·025 (Sn) |

N.B. The final values for the double experiments cannot be expressed in this way; the differences however are calculated both per g. of pigeon and per g. of snail, whether tissue or powder, as is indicated above by the letters (Pg) and (Sn).

The first experiment made it clear that the snail-hepatopancreas powder forms no uric acid when shaken alone in bicarbonate-Ringer solution, analogous to pigeon-liver powder. Pigeon-kidney slices, however, when shaken for 3 hours in Ringer solution previously in contact for 2 hours with snail-hepatopancreas powder, showed an increased formation of uric acid over the control kidney slices amounting to 1·37 mg. per g. snail powder. The same conclusion was reached in Exp. 2, where the increase was of quite the same order. But the possibility remained that the increase might be due, not to any precursor of

synthetic uric acid, but to the action of pigeon-kidney xanthine oxidase on substrates contained in the snail-hepatopancreas powder. That such substrates exist to a considerable extent in the latter was shown in another part of Exp. 2, where a preparation of xanthine oxidase from milk (by the method of Dixon and Kodama [1926]) was allowed to act on the powder. A uric acid formation of 1·69 mg. per g. powder was observed. The xanthine oxidase of the pigeon-kidney, however, might be of a much lower order of activity than the milk preparation, in which case the increases would have to be regarded as synthetic rather than oxidative. Exp. 3 demonstrated that this was not the case, and that the xanthine oxidase of the pigeon-kidney slices was capable of producing from excess substrate in equivalent time a quantity of uric acid of the same order as the apparent syntheses previously observed.

The same conclusions were more directly reached in the converse experiment (4) where snail-hepatopancreas slices were allowed to act upon Ringer solution in which pigeon-liver powder had been shaken, *i.e.* a mixture known to contain the precursor for avian uric acid. The results were quite negative. Whereas the apparent syntheses of Exps. 1 and 2 had been 36·5 and 33 % of the final values respectively, the apparent synthesis was here only 10 % of the final value, a figure too near to the limits of experimental error to be significant.

Summing up, then, it may be said that the hepatopancreas powder of the snail contains no precursor which the pigeon-kidney under the best conditions can turn into uric acid, apart from an ample supply of the substrates for the xanthine oxidase of the bird. Conversely, the precursor substance known to be present in and to be formed by pigeon-liver powder cannot be turned into uric acid at a detectable rate by the snail-hepatopancreas. Gastropod-hepatopancreas and avian kidney do not, therefore, produce more uric acid by synthesis together than alone, and the conclusion is reached that the formation of uric acid must proceed along a different path in the two phyla. Convergence in its chemical aspects appears not to imply the possession of a common mechanism.

The question then arises as to whether xanthine oxidase could be essential to the whole formation of uric acid by the snail. If the purine ring were synthesised at an earlier stage, with the formation of the less oxidised compounds, such as guanine and adenine, xanthine oxidase would deal, not only with the ammonia ultimately derived from nuclein catabolism, but also with that derived from the deamination of amino-acids. An active xanthine oxidase would therefore be required in the gastropod-hepatopancreas. The literature contains very little information on this matter, but Mendel and Wells [1909] failed to find the enzyme in the hepatopancreas of the marine gastropod, *Sycotypus canaliculatus*, although some uric acid was present (5·6 mg. per 100 g. wet weight). In the crustacean *Homarus*, xanthine oxidase was absent from all the tissues and blood, according to Morgan [1926]. It was therefore striking that in experiments designed to test its presence in the hepatopancreas of *Helix*, the result was convincingly positive.

| | mg. hepato-pancreas taken | Hours' shaking | mg./g. in 6 hours | Difference |
|---|---|---|---|---|
| *Slices* | | | | |
| No addition | 555 | 6 | 0·317 | — |
| *plus* hypoxanthine | 605 | 6 | 0·597 | +0·280 |
| *plus* xanthine | 760 | 6 | 0·626 | +0·309 |
| *Powder* | | | | |
| No addition | 188 | 4½ | 1·38 | — |
| *plus* hypoxanthine | 200 | 4½ | 1·37 | 0·0 |
| *plus* xanthine | 200 | 4½ | 1·28 | 0·0 |

(Sufficient hypoxanthine or xanthine was added to make 3·15 mg. uric acid.)

The enzyme had disappeared from the Willstätter powder, a result not surprising in view of the relative instability of the tissue-xanthine oxidases.

Further experiments on the mode of synthesis of uric acid, both in birds and gastropods, will be of great interest, and we hope to continue our work as the material becomes available. Comparison of the nitrogen catabolism of the different classes of molluscs (gastropods, lamellibranchs, cephalopods) will have particular value. Lamellibranchiate molluscs show no notable accumulation or excretion of uric acid, and their nephridial concrements are chiefly composed of calcium phosphate; "Haben wir bei den Gastropoden vor allem Harnsäure als Ausscheidungsprodukt der Emunktorien kennen gelernt, so werden wir finden, das bei den Muscheln diese Substanz als Excretionsstoff bedeutend, ja fast ganz zurücktritt, und dagegen hier positive Harnstoffbefunde zu verzeichnen sind" says Strohl [1914]. This conclusion, supported by much qualitative literature (in which perhaps the best papers are those of Letellier [1887; 1891] and Marchal [1889]), is especially significant when we remember that the Lamellibranchs are exclusively aquatic, whereas about 50 % of the Gastropod species are terrestrial (*cf.* the classification of Needham [1929]). What is the source of lamellibranch urea? Does it originate from a cyclical synthetic mechanism, or is it in all invertebrates derived from arginine alone? And of what nature is the nitrogen excretion of the purely aquatic gastropods?

## Summary.

1. An account is given of experiments designed to elucidate the sources and mode of formation of the excreted urea and uric acid in a terrestrial gastropod, the snail *Helix pomatia*. Manometric technique, using tissue slices and metabolic experiments on the intact animal, were the two main lines of approach. The peculiar difficulties inherent in this material are described in the text.

2. Only one-fortieth part of the excreted urea can be derived from the urea of the normal diet.

3. From *in vitro* experiments no evidence could be obtained of any mechanism synthesising urea from the products of deamination.

4. In general, the arginine of the diet appears to be responsible for the excreted urea. It is noteworthy that this is also the case in another terrestrial class, the birds.

5. *In vivo* experiments, however, show that additional urea excretion may follow the injection of amino-acids, especially if carbohydrate be administered simultaneously. This fact may indicate the existence of a synthetic mechanism, but its interpretation in terms of "diuresis" is at least equally plausible.

6. The discovery of arginase in *Helix* by previous workers has been confirmed. It is of much evolutionary interest that a portion of the ornithine cycle exists already in some invertebrates.

7. The discovery of urease in *Helix* by previous workers has also been confirmed.

8. The uricotelic character of the metabolism of this gastropod is supported by new *in vitro* experiments. But the hepatopancreas of the snail contains no precursor which the pigeon-kidney can convert into uric acid, apart from an ample supply of substrates for xanthine oxidase. Conversely, the precursor substance known to be present in and to be formed by pigeon-liver cannot be turned into uric acid by the snail-hepatopancreas. Thus the formation of uric acid from the ammonia of protein deamination probably follows different courses in the two phyla.

9. Xanthine oxidase is shown to be present in the hepatopancreas of the snail, though hitherto unrecorded from molluscs or crustaceans. It is suggested that this enzyme may be on the main path of formation of molluscan uric acid, whether derived ultimately from nuclein breakdown or from protein deamination. The catabolism of nucleoprotein alone cannot account for the quantities of uric acid present in the nephridium and from time to time excreted.

10. If the molluscan hepatopancreas can be homologised with the liver Clementi's rule does not apply outside the vertebrate phylum, since in *Helix* arginase and uricotelic metabolism coexist. On the other hand, Przyłęcki's rule, that uricotelic organisms never contain uricase, receives new support.

The thanks of the authors are due to Prof. H. Cardot for his frequent and generous counsel on the physiology of molluscs, to Dr G. Millikan for his estimations of the $CO_2$ of the haemolymph, to Mr N. G. Heatley for electrometric $p_H$ determinations, to Mr James Shepherd for his cryoscopic measurements, and to Dr David Green. One of us (E. B.) is indebted to the Royal Commission for the Exhibition of 1851 for a Senior Studentship during the tenure of which this work was done. Its expenses were partly defrayed by a Government Grant from the Royal Society.

## REFERENCES.

Albrecht (1920). *J. Biol. Chem.* **45**, 395.
Arvanitaki and Cardot (1932). *J. Physiol. Path. Gen.* **30**, 577.
Benedict and Franke (1922). *J. Biol. Chem.* **52**, 386.
Benzinger and Krebs (1933). *Klin. Woch.* **12**, 1206.
Bernard and Bonnet (1930). *Compt. Rend. Soc. Biol.* **103**, 1119.
Bojanus (1818). Oken's Isis.
Cardot and Troussier (1929). *Compt. Rend. Soc. Biol.* **103**, 71.
Chapheau (1932). Thesis, Bordeaux, and *Bull. Sta. Biol. d'Arcachon.*
Clementi (1915). *Arch. Fisiol.* **13**, 189.
— — (1918). *Rend. R. Acad. Lincei*, **27**, 299.
—— (1932). *Proc. XIV Congr. Internaz. Fisiol.* Rome, 24.
Clements, Howes and Wells (1933). *Nature*, **131**, 330.
Cooke (1895). Camb. Nat. Hist. Series, **3**, 242.
Delaunay (1927). *Trav. Sta. Biol. d'Arcachon*, 1.
—— (1931). *Biol. Rev.* **6**, 265.
Dixon (1934). Manometric methods. (Camb. Univ. Press.)
—— and Kodama (1926). *Biochem. J.* **20**, 1104.
Edlbacher (1925). *Z. physiol. Chem.* **148**, 273.
—— (1929). *Z. physiol. Chem.* **185**, 1.
Folin (1930). *J. Biol. Chem.* **86**, 179.
—— (1933). *J. Biol. Chem.* **101**, 111.
Fosse (1912). *Compt. Rend. Acad. Sci.* **155**, 851.
—— (1913). *Compt. Rend. Acad. Sci.* **156**, 1938.
—— (1916). *Ann. Chim.* (9), **6**, 13 and 155.
—— Brunel and de Graeve (1930). *Compt. Rend. Acad. Sci.* **190**, 79.
—— and co-workers (1929–34). *Compt. Rend. Acad. Sci.* **188** ff.
Gatenby and Duthie (1932). *J. Roy. Micr. Soc.* **52**.
—— and Hill (1934). *Quart. J. Micr. Soc.* **76**, 331.
Hesse (1910). *Z. allg. Physiol.* **10**, 273.
Hunter and Dauphinee (1924). *Proc. Roy. Soc. Lond.* B **97**, 227.
Jacobson (1820, 1). *Deutsch. Arch. Physiol.* **6**.
—— (1820, 2). *J. Physique*, **91**.
Kamada (1933). *J. Exp. Biol.* **10**, 75.
König (1903). Chem. Nahr.-Genussm. **1**, 788. (Springer, Berlin.)

Krebs (1933). In Oppenheimer's Handbuch der Biochemie, **1**, 863.
—— (1934). Ergebn. der Enzymforschung, **3**, 247 ff.
—— and Henseleit (1932). *Z. physiol. Chem.* **210**, 33.
Künkel (1916). Thesis, Heidelberg.
Letellier (1887). *Arch. Zool. Exp.* (ser. 2), **5**, bis 2.
—— (1891). *Compt. Rend. Acad. Sci.* **112**, .
Manderscheid (1933). *Biochem. Z.* **263**, 245.
Marchal (1889). *Mém. Soc. Zool. Franç.* **3**, 31.
May (1931). *Z. Biol.* **91**, 215.
—— (1932). *Z. Biol.* **92**, 319 and 325.
Mendel and Wells (1909). *Amer. J. Physiol.* **24**, 170.
Meyerhof (1930). Die Chemischen Vorgänge im Muskel. (Springer, Berlin.)
—— (1928). *Arch. Sci. Biol.* **12**, 536.
—— and Lohmann (1928). *Biochem. Z.* **196**, 22.
Morgan (1926). *Biochem. J.* **20**, 1282.
Needham, D. M., Needham, J., Baldwin and Yudkin (1932). *Proc. Roy. Soc. Lond.* B **110**, 260.
Needham, J. (1929). *Sci. Progr.* **23**, 633.
Osborne (1924). The vegetable proteins, 2nd edition. (Longmans, Green and Co., London.)
Przyłęcki (1922). *Arch. Int. Physiol.* **20**, 103.
—— (1925). *Arch. Int. Physiol.* **24**, 317.
Salaskin, Solovjev and Tjukov (1932). *Z. physiol. Chem.* **205**, 1.
Schuler and Reindel (1933). *Z. physiol. Chem.* **221**, 209, 232.
Solovjev and Mardaschev (1932). *Z. physiol. Chem.* **205**, 1.
Stahl (1889). *Jen. Z. Naturwiss.* **22**.
Stransky (1933). *Biochem. Z.* **266**, 287.
Strohl (1914). In Winterstein's Handbuch der vergl. Physiol. **2**, 443.
Sulima (1914). *Z. Biol.* **63**, 223.
Warburg (1924). *Biochem. Z.* **152**, 51.
Weber (1930). *J. Biol. Chem.* **86**, 217.
Wolf (1933). *Z. vergl. Physiol.* **19**, 1.

# CLXXXVII. THE URINARY EXCRETION OF ASCORBIC AND DEHYDROASCORBIC ACIDS IN MAN.

By SYDNEY WALGATE JOHNSON
and SYLVESTER SOLOMON ZILVA[1].

*From the Division of Nutrition, Lister Institute, London.*

(*Received June 21st, 1934.*)

The antiscorbutic activity of dehydroascorbic acid (the reversibly oxidised form of ascorbic acid) brings to the forefront the problem of the relative functions of these two active forms in animal and plant metabolism. As a contribution to this main thesis, representative experiments are described in this communication dealing with the relation between the ingestion and urinary excretion of ascorbic and dehydroascorbic acids. The investigation was carried out on four adult subjects.

Van der Walle [1922] found that a daily dose of 20 ml. of fresh normal human urine afforded no significant protection from scurvy to guinea-pigs on a scorbutic diet.

Euler and Klussman [1933] examined some specimens of urine from normal, febrile and diabetic subjects and found that they reduced indophenol to an appreciable extent.

Van Eekelen *et al.* [1933] claim that the indophenol-reducing capacity of human urine increases after the consumption of much fruit or of high doses of decitrated lemon juice. They also state that this capacity gradually disappears from the urine of guinea-pigs on a scorbutic diet. On the other hand when decitrated lemon juice is injected intravenously into rabbits the oxidised form of the vitamin is excreted in the urine in consequence.

Two more communications [Harris *et al.* 1933; Hess and Benjamin, 1934] have appeared in this connection whilst our work was in progress.

Harris *et al.* observed that the urine of normal individuals showed a surprisingly constant indophenol-reducing capacity (equivalent to 0·03 mg. of ascorbic acid per ml.) the daily output being generally in the neighbourhood of 30–33 mg. In the case of an adult subject on a scorbutic diet after ingestion of a high dose, the urinary output of ascorbic acid, as measured by the indophenol titration, rose rapidly, reaching a maximum in 3 hours after ingestion from which it quickly fell to a nearly normal level.

Hess and Benjamin, who made their observations on children, also assessed the ascorbic acid content of the urine by means of the indophenol titration. They found that the urinary output of ascorbic acid under normal conditions was low whether vitamin C was excluded from the diet or whether it was ingested in adequate amounts. Only when 1 pint of orange juice daily was given

[1] Member of the Scientific Staff, Medical Research Council.

to the children did the capacity for reducing indophenol become manifest in the urine. Even then an appreciable excretion of ascorbic acid in the urine did not appear until the fourth day, when the amount excreted rose rapidly but fell immediately the dose was discontinued.

### Experimental methods.

The ascorbic acid in the urine was determined by direct indophenol titration. The figures thus obtained were found by biological tests to be truly representative of ascorbic acid under the conditions of our experiments.

During the day the urine was titrated immediately after being passed. The night urine when pooled with the evening urine was collected over sulphuric acid and titrated on the following morning. Under these conditions the ascorbic acid excreted was found to be stable for several days. Acetic acid had a less stabilising effect and was used occasionally when only 2–3 hours elapsed between the collection and titration, for instance in the urine collected at 8 a.m.

The titrations were carried out with $N/1000$ indophenol at $p_H$ 3. At this reaction either the reduction of the indicator by substances in urine other than ascorbic acid such as phenolic compounds is suppressed, or the speed of reduction is reduced to a rate which does not seriously vitiate the end-point due to the quickly reducing ascorbic acid.

Ascorbic acid was taken in the form of orange juice when small doses (150–350 mg.) were employed and in the form of concentrated decitrated lemon juice when higher doses were required (1000 mg.). The latter was prepared by concentrating decitrated lemon juice to a small volume at low pressure at 40°, precipitating it with 96 % alcohol, filtering and concentrating the filtrate at low temperature and pressure to a suitable volume. The ascorbic acid contents of these preparations and of the orange juice were determined by direct titration with indophenol.

### Ascorbic acid in urine.

As will be seen from the observations described below the ingestion of antiscorbutic substances raised the indophenol-reducing capacity of the urine. On the other hand the urine from subjects who subsisted on diets not very rich in vitamin C or on scorbutic diets showed indophenol-reducing properties of a very low order, which at times were not even characteristic of ascorbic acid. These observations would therefore suggest that the quick reduction of the indicator by urine in acid solution was due to ascorbic acid. On reflection, however, one could not exclude the possibility of the conversion in the body of ascorbic acid into inactive or less active isomerides or degradation products which would retain the reducing properties of the original acid. To test this hypothesis the following experiment was instituted. A. E. K. received 875 mg. of ascorbic acid. After 7 hours he passed 450 ml. of urine containing 71 mg. of ascorbic acid per 100 ml. as assessed by direct indophenol titration. Similarly at a later stage of the test S. W. J. received 1000 mg. of ascorbic acid and after 5 hours passed 140 ml. of urine containing 83 mg. of the acid per 100 ml. of urine. These urines were brought to $p_H$ 3 with sulphuric acid and stored in the cold room. A. E. K.'s urine lost during storage only about 12 % of its indophenol-reducing capacity in 5 days whilst S. W. J.'s urine lost 30 % in 10 days. These urines were tested curatively for their antiscorbutic potency on guinea-pigs which had subsisted for 14 days on a scorbutic diet. An amount of urine (titrated daily) which reduced 6 ml. $N/1000$ indophenol (equivalent to

0·5 mg. of ascorbic acid) was administered each day. Fig. 1 which gives a graphic representation of the test shows that the antiscorbutic potency of the urine dose was of the same order as that of 0·5 mg. of ascorbic acid. Its indophenol-reducing capacity can therefore be ascribed mainly to this compound.

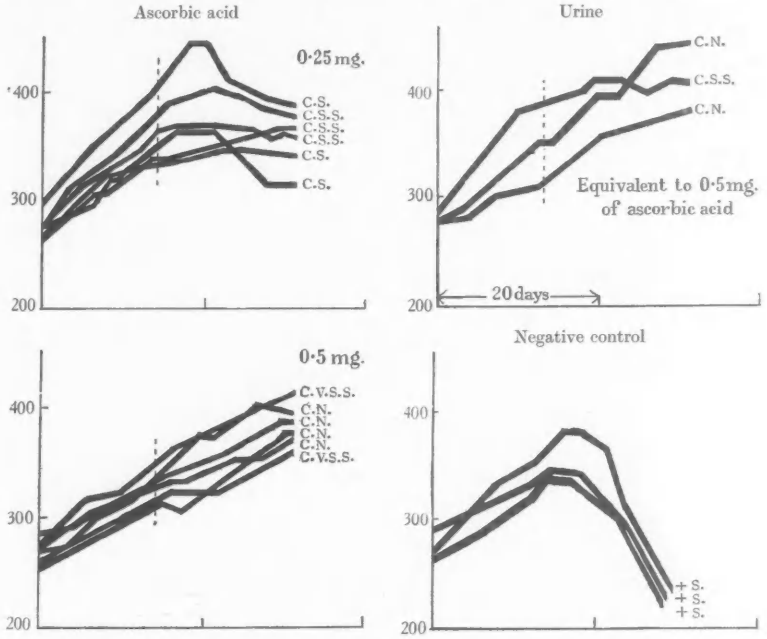



Fig. 1.

↑ = beginning of dosing.
+ = died.
C = chloroformed.
N = Normal.
V.S.S. = very slight scurvy.
S.S. = slight scurvy.
S. = scurvy.

*Preliminary.*

Before dealing with any further results, it is necessary to record the "normal" indophenol-reducing properties of the urines of the four experimental subjects concerned in this investigation. Three of these, S. W. J., A. E. K. and H. H., while using ordinary mixed diets on which they usually subsist during the winter passed urine which for days scarcely reduced indophenol. Even when the titrations were significant the output of ascorbic acid per 24 hours was of the order of 10–15 mg. During the summer, as was to be expected, the indophenol-reducing capacity rose to some extent. The fourth subject (S. S. Z.) for reasons not connected with the present inquiry used a diet in which bread and potatoes were replaced by green vegetables; in addition he consumed regularly two oranges a day. In consequence his "normal" urinary output of ascorbic acid was 80–150 mg. per day even during the winter. At no time in the period of 8 months during which the experiments described in this communication were carried out were there any indications even of latent scurvy in any of the four subjects.

*Conditions under which ascorbic acid is excreted in the urine.*

It soon became evident from exploratory experiments that the appearance of ascorbic acid in the urine after its ingestion was dependent not only on the magnitude of the dose but also on the previous dietetic history of the subject in respect of vitamin C. The subject had to be "saturated" with the vitamin before his level of excretion in the urine became constant. The time taken to attain this was controlled by the degree of "saturation" of the subject at the beginning of the experiment and by the size of the daily dose. This is exemplified by the following experiments. S. W. J. was subsisting on a diet low in vitamin C which consisted mainly of eggs, cheese, fried bacon and bread with small quantities of meat, fish and potatoes. On this regimen his urine did not show any capacity for reducing indophenol for a number of consecutive days. The small amount of vitamin C contained in this diet exercised no influence on the results, since similar ones were obtained in the case of S. S. Z. on a strictly scorbutic diet. On successive days at the beginning of the experiment S. W. J. took, 30, 30, 60, 60, 90 and 90 mg. ascorbic acid respectively. No sample of urine, which was collected at frequent intervals, showed any significant capacity for reducing indophenol. Only after the seventh day were significant figures obtained. Table I gives the data from this day to the eighteenth day of the experiment. The figures show that the amount of ascorbic acid returned in the urine

Table I.

| | Intake | | Excretion in urine | | | | |
|---|---|---|---|---|---|---|---|
| Day | Time dose taken | Ascorbic acid mg. | Time urine passed | Volume of urine ml. | Ascorbic acid per 100 ml. mg. | Total amount of ascorbic acid mg. | Ascorbic acid output per hour mg. |
| 7th | 10.30 a.m. | 120 | 12.45 p.m. | 200 | 0·4 | 0·8 | 0·3 |
| | | | 2.00 | 200 | 0·4 | 0·8 | 0·6 |
| | | | 4.30 | 270 | 0·8 | 2·3 | 0·9 |
| | | | 5.30 | 60 | 0·8 | 0·5 | 0·5 |
| | | | 8.00 a.m. | 1010 | 0·8 | 8·4 | 0·6 |
| | | | 10.15 | 110 | 0·8 | 0·9 | 0·4 |
| | | | Total | **1850** | — | **13·7** | — |
| 8th | 12.15 p.m. | 120 | 1.00 p.m. | 180 | 0·8 | 1·5 | 0·5 |
| | | | 2.00 | 70 | 1·7 | 1·2 | 1·2 |
| | | | 3.45 | 140 | 6·3 | 8·8 | 5·0 |
| | | | 5.45 | 100 | 6·7 | 6·7 | 3·4 |
| | | | 8.00 a.m. | 880 | 1·3 | 11·0 | 0·8 |
| | | | 10.15 | 110 | 0·8 | 0·9 | 0·4 |
| | | | Total | **1480** | — | **30·1** | — |
| 9th | 10.30 a.m. | 85 | 11.30 a.m. | 100 | 1·7 | 1·7 | 1·4 |
| | | | 12.30 p.m. | 200 | — | — | — |
| | | | 2.00 | 180 | 1·3 | 2·3 | 1·5 |
| | | | 3.00 | 205 | 0·8 | 1·7 | 1·7 |
| | | | 5.15 | 170 | 1·7 | 2·8 | 1·2 |
| | | | 6.15 | 70 | 1·7 | 1·0 | 1·0 |
| | | | 8.00 a.m. | 930 | 0·8 | 7·8 | 0·6 |
| | | | 11.30 | 170 | 1·7 | 2·8 | 0·8 |
| | | | 2.00 p.m. | 210 | 1·7 | 3·5 | 1·4 |
| | | | Total | **2235** | — | **23·6** | — |
| 10th | 2.45 p.m. | 100 | 8.00 a.m. | 1270 | 2·5 | 31·8 | 1·8 |
| | | | 10.30 | 80 | 1·7 | 1·3 | 0·5 |
| | | | Total | **1350** | — | **33·1** | — |

Table I (*cont.*).

| | Intake | | Excretion in urine | | | | |
|---|---|---|---|---|---|---|---|
| Day | Time dose taken | Ascorbic acid mg. | Time urine passed | Volume of urine ml. | Ascorbic acid per 100 ml. mg. | Total amount of ascorbic acid mg. | Ascorbic acid output per hour mg. |
| 11th | 11.00 a.m. | 130 | 11.30 a.m. | 130 | 5·0 | 6·5 | 3·3 |
| | | | 12.30 p.m. | 630 | 5·8 | 36·8 | 3·3 |
| | | | 8.00 a.m. | 480 | 1·7 | 8·0 | 1·0 |
| | | | Total | **1240** | — | **51·3** | — |
| 12th | 8.30 a.m. | 120 | 6.00 p.m. | 770 | 3·3 | 25·7 | 2·6 |
| | | | 8.00 a.m. | 465 | 2·5 | 11·6 | 0·8 |
| | | | 10.15 | 60 | 1·7 | 1·0 | 0·4 |
| | | | Total | **1295** | — | **38·3** | — |
| 13th | 11.00 a.m. | 90 | 1.15 p.m. | 120 | 1·7 | 2·0 | 0·7 |
| | | | 4.00 | 140 | 6·7 | 9·3 | 3·4 |
| | | | 5.45 | 130 | 2·5 | 3·3 | 1·9 |
| | | | 8.00 a.m. | 720 | 1·7 | 12·0 | 0·8 |
| | | | 10.30 | 120 | 1·7 | 2·0 | 0·8 |
| | | | 12.15 p.m. | 175 | 2·5 | 4·4 | 2·5 |
| | | | Total | **1405** | — | **33·0** | — |
| 14th | 12.30 p.m. | 120 | 2.15 p.m. | 100 | 7·5 | 7·5 | 3·8 |
| | | | 8.00 a.m. | 800 | 5·0 | 40·0 | 2·3 |
| | | | 10.30 | 110 | 2·5 | 2·8 | 1·1 |
| | | | 12.30 p.m. | 155 | 2·5 | 3·9 | 2·0 |
| | | | Total | **1165** | — | **54·2** | — |
| 15th | 12.30 p.m. | 120 | 2.15 p.m. | 160 | 4·1 | 6·7 | 3·8 |
| | | | 5.00 | 140 | 18·3 | 25·7 | 9·3 |
| | | | 8.00 a.m. | 860 | 3·3 | 28·7 | 1·9 |
| | | | 10.30 | 90 | 6·7 | 6·0 | 2·4 |
| | | | Total | **1250** | — | **67·1** | — |
| 16th | 12.30 p.m. | 120 | 1.00 p.m. | 170 | 5·0 | 8·5 | 3·4 |
| | | | 3.30 | 220 | 11·7 | 25·7 | 10·3 |
| | | | 4.30 | 180 | 11·7 | 21·0 | 21·0 |
| | | | 8.00 a.m. | 1010 | 4·1 | 42·1 | 2·7 |
| | | | 10.30 | 160 | 5·0 | 8·0 | 3·1 |
| | | | Total | **1740** | — | **105·3** | — |
| 17th | 12.30 p.m. | 150 | 1.00 p.m. | 120 | 5·0 | 6·0 | 2·4 |
| | | | 3.15 | 220 | 18·3 | 40·3 | 17·9 |
| | | | 5.15 | 165 | 21·7 | 37·8 | 18·9 |
| | | | 8.00 a.m. | 710 | 7·5 | 53·3 | 3·4 |
| | | | Total | **1215** | — | **137·4** | — |
| 18th | 10.30 a.m. | 150 | 11.00 a.m. | 80 | 12·5 | 10·0 | 3·3 |
| | | | 12.30 p.m. | 70 | 22·9 | 15·1 | 10·0 |
| | | | 8.00 a.m. | 1260 | 8·3 | 105·1 | 5·4 |
| | | | Total | **1410** | — | **130·2** | — |

gradually rose to a level which remained constant after the seventeenth day. This suggested that the subject was "saturated" with ascorbic acid, but in order to insure complete "saturation" he took a further 1000 mg. of which 307 mg. was returned during the first, and only 45 mg. during the second, 24 hours. 150 mg. of ascorbic acid were then taken. Full details of the intake and urinary excretion of ascorbic acid *etc.* during this and the subsequent 9 days are given in Table II. It will be seen that in this part of the experiment the return of ascorbic acid in the urine after discontinuing the dose fell to very low values in a few days. In contrast to what happened in the initial stages of the

Table II.

| | Intake | | Excretion in urine | | | | |
|---|---|---|---|---|---|---|---|
| Day | Time dose taken | Ascorbic acid mg. | Time urine passed | Volume of urine ml. | Ascorbic acid per 100 ml. mg. | Total amount of ascorbic acid mg. | Ascorbic acid output per hour mg. |
| 1st | 12.45 p.m. | 150 | 3.30 p.m. | 140 | 25·0 | 35·0 | 12·7 |
| | | | 5.00 | 85 | 29·1 | 24·8 | 16·5 |
| | | | 8.00 a.m. | 800 | 8·3 | 66·7 | 4·5 |
| | | | 10.30 | 90 | 8·3 | 7·5 | 3·0 |
| | | | 12.00 noon | 90 | 3·3 | 3·0 | 2·0 |
| | | | 1.0 p.m. | 70 | 2·5 | 1·8 | 1·8 |
| | | | Total | **1275** | — | **138·8** | — |
| 2nd | 12.45 p.m. | | 5.45 p.m. | 180 | 6·7 | 12·0 | 2·5 |
| | | | 8.00 a.m. | 620 | 3·3 | 20·7 | 1·4 |
| | | | 10.45 | 120 | 4·1 | 5·0 | 1·8 |
| | | | 12.15 p.m. | 90 | 1·7 | 1·5 | 1·0 |
| | | | 1.15 | 160 | 1·7 | 2·7 | 2·7 |
| | | | Total | **1170** | — | **41·9** | — |
| 3rd | 12.45 p.m. | | 4.30 p.m. | 150 | 5·0 | 7·5 | 2·3 |
| | | | 8.00 a.m. | 900 | 0·8 | 7·5 | 0·5 |
| | | | 10.30 | 150 | 0·8 | 1·3 | 0·5 |
| | | | Total | **1200** | — | **16·3** | — |
| 4th | 12.45 p.m. | | 2.30 p.m. | 210 | 0·8 | 1·8 | 0·5 |
| | | | 8.00 a.m. | 1270 | 0·4 | 5·3 | 0·3 |
| | | | Total | **1480** | — | **7·1** | — |
| 5th | 12.45 p.m. | | 8.30 a.m. | 1300 | 0·4 | 5·4 | 0·2 |
| | | | 10.30 | 180 | 0·4 | 0·8 | 0·4 |
| | | | Total | **1480** | — | **6·2** | — |
| 6th | 12.45 p.m. | | 4.30 p.m. | 450 | 0·4 | 1·8 | 0·3 |
| | | | 8.00 a.m. | 800 | 0·4 | 3·2 | 0·2 |
| | | | 10.30 | 130 | 0·4 | 0·5 | 0·2 |
| | | | Total | **1380** | — | **5·5** | — |
| 7th | 12.00 noon | 180 | 2.30 p.m. | 155 | 16·0 | 24·8 | 6·2 |
| | | | 4.30 | 130 | 30·0 | 39·0 | 19·5 |
| | | | 5.30 | 75 | 11·7 | 8·8 | 8·8 |
| | | | 8.00 a.m. | 1450 | 4·1 | 60·4 | 4·2 |
| | | | 10.30 | 110 | 8·3 | 9·1 | 3·6 |
| | | | 11.30 | 110 | 3·3 | 3·6 | 3·6 |
| | | | Total | **2030** | — | **145·7** | — |
| 8th | 12.00 noon | | 1.00 p.m. | 100 | 3·3 | 3·3 | 2·2 |
| | | | 3.15 | 110 | 5·8 | 6·4 | 2·8 |
| | | | 5.45 | 110 | 5·8 | 6·4 | 2·5 |
| | | | 8.00 a.m. | 650 | 1·7 | 10·8 | 0·8 |
| | | | 10.00 | 110 | 1·7 | 1·9 | 0·8 |
| | | | 11.30 | 130 | 4·1 | 5·4 | 3·6 |
| | | | Total | **1210** | — | **34·2** | — |
| 9th | 12.00 noon | | 8.00 a.m. | 1200 | 1·7 | 20·0 | 1·0 |
| | | | 10.30 | 110 | 1·7 | 1·9 | 0·8 |
| | | | Total | **1310** | — | **21·9** | — |
| 10th | 12.00 noon | | 2.30 p.m. | 140 | 2·5 | 3·5 | 0·9 |
| | | | 6.00 | 180 | 1·7 | 3·0 | 0·8 |
| | | | 9.15 | 210 | 1·7 | 3·5 | 1·0 |
| | | | 8.00 a.m. | 800 | 0·8 | 6·7 | 0·6 |
| | | | 11.30 | 180 | 1·7 | 3·0 | 0·8 |
| | | | Total | **1510** | — | **19·7** | — |

experiment (Table I) however the level of excretion could now be raised by the administration of a single dose of 150 mg. or 180 mg. to that of the previously obtained constant level. In other words when the subject is "saturated" this level is attained at once, but on the other hand many days may elapse in cases where the vitamin C store is depleted. This phenomenon has also been observed in the other three subjects and the results are in agreement with those obtained by Hess and Benjamin on children. Our results further suggest that the maximum rate of excretion of ascorbic acid invariably occurred about the fourth hour after ingestion and that the initial rate was reached again within about 24 hours. Harris *et al.* also observed that the maximum rate of excretion occurred after a similar interval. It may be further noted that the concentration of ascorbic acid in the urine at any time is controlled by the amount of water excreted, but, as will be amplified later, its rate of excretion is independent of diuresis. The concentration of ascorbic acid in urine is therefore by itself of no great significance.

*Urinary excretion of ascorbic acid after ingestion of high doses.*

It will be remembered that as S. W. J. became "saturated," about 80 % of the ingested ascorbic acid appeared in the urine when the dose taken was of the order of 150 mg. When, on the other hand, the dose was raised to 1000 mg. only about 35 % of the acid was returned in the urine during the subsequent 48 hours. This observed disparity in the relative proportions of ascorbic acid "retained" on high or low doses called for further investigation and it was subsequently shown that the lower percentage of excretion is indeed characteristic of high doses. This is illustrated by the following typical experiments.

S. S. Z. subsisting on his "normal" diet on which, as has already been mentioned, he excreted 70–150 mg. of ascorbic acid, took a daily dose of the order of 1000 mg. on six consecutive days. The urine was titrated at each collection during the 24 hour period. It will be seen from Table III that, even if his basal output of about 100 mg. *per diem* is ignored, only 45 % of the ingested ascorbic acid was accounted for in the urine. Furthermore, during the 4 days immediately following this period of high dosing he excreted 117, 114, 110 and 77·3 mg. of ascorbic acid respectively, thus returning immediately to his basal level of urinary excretion of the vitamin. According to these figures about 400–600 mg. of the acid were not accounted for in this case as against about 25 mg. "retained" by S. W. J. on the low dose.

Whilst the above is illustrative of a "saturated" case, the following experiment deals with a subject who was not previously "saturated." A. E. K. subsisted for a long time on a diet low in vitamin C which was constituted similarly to the one used by S. W. J. in the experiment previously described. He received high doses of ascorbic acid on each of nine successive days. The results which are given in Table IV in an abridged form show that prior to the high dosage he returned only 10·5 mg. of the acid in the urine during 24 hours. The level then rose until the fourth day, after which it became more or less constant. Immediately following this period of high dosing the urinary excretion of ascorbic acid fell rapidly, and on the third day reached the low level of 5·5 mg. for the 24 hours.

This case differs from that of S. S. Z. in that being "unsaturated" he took several days to reach the relatively constant maximum level of output. On the other hand, as might be expected, it took very much less time to "saturate" A. E. K. on high doses than it did S. W. J. on low doses (Table I). Yet the return to the original level in all three cases occurred in approximately the same time.

## Table III.

Dose taken at 8.00 a.m. on each day.

| | | | Excretion in urine | | | | |
|---|---|---|---|---|---|---|---|
| Day | Intake. Ascorbic acid mg. | Time urine passed | Volume of urine ml. | Ascorbic acid per 100 ml. mg. | Total amount of ascorbic acid mg. | Ascorbic acid output per hour mg. | Ascorbic acid output as percentage of intake |
| 1st | 1125 | 10.30 a.m. | 130 | 58·3 | 75·8 | 30·0 | |
| | | 3.15 p.m. | 270 | 79·0 | 213·8 | 45·0 | |
| | | 5.30 | 160 | 29·0 | 47·0 | 20·0 | |
| | | 12.00 midnight | 385 | 20·0 | 77·0 | 11·8 | |
| | | 8.00 a.m. | 660 | 13·3 | 88·0 | 11·0 | |
| | | Total | **1605** | — | **501·6** | — | **44·5** |
| 2nd | 875 | 11.00 a.m. | 120 | 116·7 | 140·0 | 46·7 | |
| | | 3.15 p.m. | 230 | 54·1 | 124·4 | 19·5 | |
| | | 5.30 | 90 | 41·6 | 37·5 | 16·7 | |
| | | 12.00 midnight | 430 | 20·0 | 86·0 | 13·0 | |
| | | 8.00 a.m. | 740 | 11·0 | 80·0 | 10·0 | |
| | | Total | **1610** | — | **467·9** | — | **48·6** |
| 3rd | 1083 | 8.00 a.m. to 12.00 midnight | 930 | 43·7 | 407·0 | 25·4 | |
| | | 12.00 midnight to 8.00 a.m. | 750 | 10·0 | 75·0 | 9·3 | |
| | | Total | **1680** | — | **482·0** | — | **44·5** |
| 4th | 1083 | 8.00 a.m. to 12.00 midnight | 950 | 31·2 | 297·0 | 18·6 | |
| | | 12.00 midnight to 8.00 a.m. | 450 | 15·0 | 67·5 | 8·4 | |
| | | Total | **1400** | — | **364·5** | — | **33·6** |
| 5th | 1167 | 10.15 a.m. | 180 | 33·3 | 60·0 | 26·7 | |
| | | 3.15 p.m. | 305 | 62·5 | 190·6 | 38·1 | |
| | | 12.00 midnight | 490 | 36·7 | 179·7 | 20·5 | |
| | | 8.00 a.m. | 750 | 15·0 | 112·5 | 14·0 | |
| | | Total | **1725** | — | **542·8** | — | **46·5** |
| 6th | 917 | 10.15 a.m. | 140 | 50·0 | 70·0 | 31·2 | |
| | | 2.30 p.m. | 280 | 62·5 | 175·0 | 41·1 | |
| | | 5.30 | 250 | 41·7 | 104·0 | 34·7 | |
| | | 12.00 midnight | 275 | 45·2 | 126·0 | 19·4 | |
| | | 8.00 a.m. | 790 | 10·0 | 79·0 | 10·0 | |
| | | Total | **1735** | — | **554·0** | — | **60·4** |

## Table IV.

| | | Excretion in urine | | | | |
|---|---|---|---|---|---|---|
| Day | Intake of ascorbic acid mg. | Volume of urine ml. | Total ascorbic acid mg. | Ascorbic acid output as percentage of intake | Maximum output per hour mg. | Minimum output per hour mg. |
| 1st | — | 630 | 10·5 | — | 0·6 | 0·2 |
| 2nd | 916 | 1715 | 77·0 | 8 | 8·0 | 0·3 |
| 3rd | 933 | 1455 | 303·0 | 30 | 31·5 | 1·1 |
| 4th | 1000 | 1100 | 402·8 | 40 | 55·0 | 1·2 |
| 5th | 800 | 2135 | 492·8 | 60 | 50·3 | 4·5 |
| 6th | 867 | 1395 | 537·5 | 61 | 75·0 | 3·6 |
| 7th | 750 | 1865 | 391·8 | 52 | 48·9 | 2·6 |
| 8th | 833 | 2025 | 476·8 | 57 | 71·1 | 3·6 |
| 9th | 875 | 1050 | 432·1 | 50 | 62·2 | 2·1 |
| 10th | 875 | 1280 | 529·5 | 60 | 111·1 | 1·7 |
| 11th | — | 1405 | 68·2 | — | 3·7 | 1·2 |
| 12th | — | 810 | 22·8 | — | 1·7 | 0·4 |
| 13th | — | 890 | 5·5 | — | 0·5 | 0·3 |

It would therefore seem that there is a definite capacity for "saturation" which is independent of the dose. Further, in the case of the high doses, the amount of ascorbic acid not accounted for in the urine is very high and apparently differs with the individual, since in the case of S. S. Z. it was of the order of 400–600 mg. whilst in that of A. E. K. it was only about 300 mg.

*The retention of ascorbic acid.*

The preceding experiments make it clear that the balance between the amount of ascorbic acid ingested and that returned in the urine cannot be taken as a true index of retention of the acid for the actual physiological function of the vitamin, since this balance depends on the dose taken. Exploratory experiments were nevertheless carried out in order to ascertain whether it was possible to obtain by this technique using low doses a rough estimate of the daily minimum amount of ascorbic acid needed. The experiments to be described show that this is unlikely since it was found that the same individual manifested two levels of urinary excretion of ascorbic acid on the same dose at different times and conversely the same level of output may occur after the ingestion of doses of different magnitude. This is illustrated by the following two experiments.

S. S. Z. was subsisting on a scorbutic diet consisting of eggs, cheese, mushrooms, bacon, chocolate and boiled sweets. Between the morning meal taken about 8 a.m. and the evening meal taken about 7 p.m. no food except tea and milk was consumed. This subject was "saturated," but before beginning the experiment he remained on the scorbutic diet without any vitamin C addition for several days till the urinary excretion fell to a low level. He then ingested 150 mg. of ascorbic acid every 24 hours. It took him 2 days (Table V) to reach the constant level of excretion of about 60 mg. which was maintained for 4 days. At this stage S. S. Z. went on a mixed diet for a few days after which he took in addition 1000 mg. of ascorbic acid to ensure full saturation and returned to the scorbutic diet for another 4 days; the level of urinary excretion reached this time was about 100 mg. for 24 hours (Table VI). After 4 days on a mixed diet the scorbutic diet was resumed for another 10 days; the level of about 100 mg. per 24 hours was consistently maintained.

S. W. J., using the previously mentioned diet low in vitamin C, "saturated" himself with ascorbic acid by ingesting standardised amounts of orange juice. His intake of ascorbic acid per day during the first 5 days of the experiment was 350 mg. of which he returned about 220 mg. daily in the urine (Table VII). On the sixth day his daily dose was reduced to 300 mg. and was taken in two portions of 150 mg. each, at midday and midnight respectively. Under these conditions it will be seen that on the whole the same daily amount was returned in the urine as with the higher dose. It may be noted that in the second part of the experiment, when the daily dose was taken in two portions, with the exception of one day, a higher percentage of the intake was returned in the urine during the period between midday and midnight than in the succeeding 12 hour period.

*The fate of ingested dehydroascorbic acid.*

The urinary excretion when dehydroascorbic acid was ingested in excess of the possible requirements of the body was next studied. Experiments described below supply definite proof that the dehydrogenated acid is reduced in the body.

Since the oxidation of ascorbic acid with indophenol or iodine would have made the preparations unsuitable for internal consumption and further since

Table V.

| | Intake | | Excretion in urine | | | | |
|---|---|---|---|---|---|---|---|
| Day | Time dose taken | Ascorbic acid mg. | Time urine passed | Volume of urine ml. | Ascorbic acid per 100 ml. mg. | Total amount of ascorbic acid mg. | Ascorbic acid output per hour mg. |
| 1st | 11.30 a.m. | 150 | 1.00 p.m. | 80 | 4·0 | 3·2 | 2·2 |
| | | | 2.00 | 85 | 5·7 | 4·9 | 4·9 |
| | | | 3.00 | 135 | 4·0 | 5·4 | 5·4 |
| | | | 4.00 | 245 | 2·1 | 5·3 | 5·3 |
| | | | 5.00 | 205 | 2·5 | 5·1 | 5·1 |
| | | | 10.00 | 315 | 4·5 | 14·2 | 2·5 |
| | | | 8.00 a.m. | 900 | 0·8 | 7·5 | 0·9 |
| | | | 10.00 | 155 | 1·8 | 2·8 | 1·4 |
| | | | Total | **2120** | — | **48·4** | — |
| 2nd | 10.00 a.m. | 150 | 11.00 a.m. | 105 | 3·3 | 3·5 | 3·5 |
| | | | 12.00 noon | 140 | 2·7 | 3·7 | 3·7 |
| | | | 1.00 p.m. | 200 | 2·7 | 5·3 | 5·3 |
| | | | 2.00 | 430 | 1·7 | 7·3 | 7·3 |
| | | | 3.00 | 80 | 4·1 | 3·3 | 3·3 |
| | | | 4.00 | 260 | 1·0 | 2·6 | 2·6 |
| | | | 5.00 | 210 | 1·5 | 3·3 | 3·3 |
| | | | 10.00 | 330 | 2·5 | 8·3 | 1·7 |
| | | | 8.00 a.m. | 1120 | 0·8 | 9·3 | 0·9 |
| | | | 10.00 | 170 | 1·7 | 2·8 | 1·4 |
| | | | Total | **3045** | — | **49·4** | — |
| 3rd | 10.00 a.m. | 150 | 11.00 a.m. | 135 | 3·5 | 4·7 | 4·7 |
| | | | 12.00 noon | 120 | 4·1 | 5·0 | 5·0 |
| | | | 1.00 p.m. | 155 | 4·0 | 6·2 | 6·2 |
| | | | 2.00 | 330 | 2·7 | 8·8 | 8·8 |
| | | | 3.00 | 315 | 1·7 | 5·2 | 5·2 |
| | | | 4.00 | 355 | 1·5 | 5·6 | 5·6 |
| | | | 5.00 | 410 | 1·7 | 6·8 | 6·8 |
| | | | 10.00 | 375 | 1·3 | 5·0 | 1·0 |
| | | | 8.00 a.m. | 1120 | 0·8 | 9·3 | 0·9 |
| | | | 10.00 | 235 | 1·8 | 4·2 | 2·1 |
| | | | Total | **3550** | — | **60·8** | — |
| 4th | 10.00 a.m. | 150 | 11.00 a.m. | 135 | 3·0 | 4·0 | 4·0 |
| | | | 12.00 noon | 255 | 2·0 | 5·1 | 5·1 |
| | | | 2.00 p.m. | 170 | 6·7 | 11·3 | 5·7 |
| | | | 5.30 | 235 | 6·7 | 15·6 | 4·5 |
| | | | 10.30 | 280 | 3·8 | 10·7 | 2·1 |
| | | | 9.30 a.m. | 1120 | 1·5 | 15·8 | 1·4 |
| | | | Total | **2195** | — | **62·5** | — |
| 5th | 9.30 a.m. | 150 | 11.00 a.m. | 140 | 5·8 | 8·2 | 5·5 |
| | | | 2.00 p.m. | 215 | 5·3 | 11·4 | 3·8 |
| | | | 6.00 | 150 | 6·7 | 10·0 | 2·5 |
| | | | 10.00 | 255 | 5·0 | 12·8 | 3·2 |
| | | | 8.00 a.m. | 875 | 1·5 | 13·3 | 1·3 |
| | | | 10.00 | 130 | 3·5 | 4·5 | 2·3 |
| | | | Total | **1765** | — | **60·2** | — |
| 6th | 10.00 a.m. | 50 | 11.00 a.m. | 130 | 3·3 | 4·3 | 4·3 |
| | | | 12.00 noon | 190 | 2·7 | 5·0 | 5·0 |
| | | | 2.00 p.m. | 305 | 2·7 | 8·1 | 4·0 |
| | | | 3.00 | 110 | 2·5 | 2·8 | 2·8 |
| | 3.00 p.m. | 50 | 4.00 | 130 | 2·3 | 3·0 | 3·0 |
| | | | 5.00 | 335 | 1·8 | 6·1 | 6·1 |
| | | | 8.00 | 370 | 3·7 | 13·5 | 4·4 |
| | 10.00 p.m. | 50 | 8.00 a.m. | 700 | 2·0 | 14·0 | 1·4 |
| | | | 10.00 | 145 | 3·8 | 5·7 | 2·9 |
| | Total | **150** | — | **2415** | — | **62·5** | — |

## Table VI.

The dose was taken at 10.00 a.m. on each day except the first day when it was taken at noon.

| | Intake | | Excretion in urine | | | | |
|---|---|---|---|---|---|---|---|
| Day | Diet | Amount of ascorbic acid mg. | Total amount of urine ml. | Total amount of ascorbic acid mg. | Maximum output of ascorbic acid per hour mg. | Time taken to reach maximum hours | Minimum output of ascorbic acid per hour mg. |
| 1st | Scorbutic | 150 | 2545 | 111·2 | 12·8 | 5 | 1·7 |
| 2nd | ,, | 150 | 2470 | 105·0 | 17·3 | 4 | 1·6 |
| 3rd | ,, | 150 | 2625 | 105·7 | 16·3 | 4 | 1·5 |
| 4th | ,, | 150 | 2510 | 101·0 | 11·7 | 4 | 1·9 |
| 5th | Mixed | No dose taken, no urine collected | | | | | |
| 6th | ,, | | | | | | |
| 7th | ,, | | | | | | |
| 8th | ,, | | | | | | |
| 9th | Scorbutic | 150 | 2055 | 183·7 | 21·6 | 4 | 3·5 |
| 10th | ,, | 150 | 1535 | 142·3 | 13·6 | 4 | 2·5 |
| 11th | ,, | 150 | 1795 | 109·8 | 15·2 | 4 | 1·8 |
| 12th | ,, | 150 | 2090 | 88·9 | 10·1 | 4 | 1·7 |
| 13th | ,, | 150 | 2155 | 98·7 | 9·4 | 4 | 1·8 |
| 14th | ,, | 150 | 2510 | 103·1 | 12·7 | 4 | 1·5 |
| 15th | ,, | 150 | 2310 | 103·8 | 12·8 | 4 | 1·5 |
| 16th | ,, | 150 | 2215 | 97·4 | 11·7 | 3 | 1·9 |
| 17th | ,, | 150 | 2360 | 101·6 | 14·4 | 4 | 1·4 |
| 18th | ,, | 150 | 2150 | 98·8 | 16·7 | 4 | 2·0 |

## Table VII.

| | Intake | | Excretion in urine | | | | |
|---|---|---|---|---|---|---|---|
| Day | Time dose taken | Ascorbic acid mg. | Time urine passed | Volume of urine ml. | Ascorbic acid per 100 ml. mg. | Total amount of ascorbic acid mg. | Ascorbic acid output per hour mg. |
| 1st | 5.15 p.m. | 350 | 8.00 a.m. | 780 | 20·8 | 162·5 | 12·2 |
| | | | 10.00 | 120 | 8·3 | 10·0 | 5·0 |
| | | | 12.00 noon | 110 | 11·0 | 11·9 | 6·0 |
| | | | 2.30 p.m. | 130 | 10·0 | 13·0 | 5·0 |
| | | | 4.30 | 70 | 21·7 | 15·0 | 7·5 |
| | | | Total | **1210** | — | **212·4** | — |
| 2nd | 5.30 p.m. | 350 | 6.30 p.m. | 120 | 6·7 | 8·0 | 4·0 |
| | | | 8.00 a.m. | 710 | 23·3 | 165·7 | 14·4 |
| | | | 10.00 | 100 | 13·3 | 13·3 | 6·0 |
| | | | 12.00 noon | 120 | 11·7 | 14·0 | 7·0 |
| | | | 3.00 p.m. | 190 | 6·7 | 12·7 | 4·2 |
| | | | 4.30 | 60 | 16·7 | 10·0 | 6·7 |
| | | | Total | **1300** | — | **223·7** | — |
| 3rd | 5.00 p.m. | 350 | 6.15 p.m. | 120 | 21·7 | 26·0 | 14·8 |
| | | | 8.00 a.m. | 780 | 21·7 | 169·0 | 12·3 |
| | | | 10.30 | 90 | 13·3 | 12·0 | 5·0 |
| | | | 1.30 p.m. | 200 | 6·7 | 13·3 | 4·4 |
| | | | Total | **1190** | — | **220·3** | — |
| 4th | 5.00 p.m. | 350 | Urine not collected on this day | | | | |
| 5th | 4.30 p.m. | 350 | 8.00 a.m. | 920 | 16·7 | 153·3 | 10·0 |
| | | | 10.30 | 100 | 5·0 | 5·0 | 2·0 |
| | | | 12.30 p.m. | 120 | 3·3 | 4·0 | 2·0 |
| | | | Total | **1140** | — | **162·3** | — |

Table VII (*cont.*).

| | Intake | | Excretion in urine | | | | |
|---|---|---|---|---|---|---|---|
| Day | Time dose taken | Ascorbic acid mg. | Time urine passed | Volume of urine ml. | Ascorbic acid per 100 ml. mg. | Total amount of ascorbic acid mg. | Ascorbic acid output per hour mg. |
| 6th | 12.15 p.m. | 150 | 2.30 p.m. | 130 | 11·7 | 15·0 | 7·5 |
| | | | 6.15 | 170 | 28·3 | 48·0 | 12·8 |
| | | | 12.00 | 220 | 18·3 | 43·3 | 7·0 |
| | | | Total for 1st 12 hours | **520** | — | **106·3** | — |
| | 12.00 midnight | 150 | 8.00 a.m. | 220 | 43·8 | 96·3 | 12·0 |
| | | | 10.45 | 60 | 20·8 | 12·5 | 4·5 |
| | | | 12.00 noon | 40 | 15·0 | 6·0 | 4·5 |
| | | | Total for 2nd 12 hours | **320** | — | **114·8** | — |
| | | | Total for 24 hours | **840** | — | **221·0** | — |
| 7th | 12.15 p.m. | 150 | 4.00 p.m. | 105 | 45·8 | 48·1 | 12·0 |
| | | | 11.30 | 240 | 21·7 | 52·1 | 7·0 |
| | | | Total for 1st 12 hours | **345** | — | **100·2** | — |
| | 11.45 p.m. | 150 | 8.00 a.m. | 200 | 17·5 | 35·0 | 4·1 |
| | | | 11.45 | 130 | 18·3 | 24·0 | 6·4 |
| | | | Total for 2nd 12 hours | **330** | — | **59·0** | — |
| | | | Total for 24 hours | **675** | — | **159·2** | — |
| 8th | 12.00 noon | 150 | 3.00 p.m. | 140 | 33·3 | 46·7 | 14·3 |
| | | | 5.30 | 110 | 29·1 | 32·0 | 12·8 |
| | | | 11.30 | 270 | 8·3 | 22·5 | 3·4 |
| | | | Total for 1st 12 hours | **520** | — | **101·2** | — |
| | 11.45 p.m. | 150 | 8.00 a.m. | 250 | 13·3 | 33·3 | 3·9 |
| | | | 10.00 | 90 | 11·7 | 10·5 | 5·2 |
| | | | 12.00 noon | 165 | 8·3 | 13·8 | 7·0 |
| | | | Total for 2nd 12 hours | **505** | — | **57·6** | — |
| | | | Total for 24 hours | **1025** | — | **158·8** | — |
| 9th | 12.15 p.m. | 150 | 1.00 p.m. | 100 | 12·5 | 12·5 | 12·5 |
| | | | 2.15 | 105 | 25·0 | 26·3 | 21·0 |
| | | | 4.15 | 100 | 46·7 | 46·7 | 46·7 |
| | | | 5.30 | 90 | 13·3 | 12·0 | 10·0 |
| | | | 11.30 | 250 | 8·3 | 20·8 | 3·5 |
| | | | Total for 1st 12 hours | **645** | — | **118·3** | — |
| | 11.45 p.m. | 150 | 8.00 a.m. | 300 | 16·7 | 50·0 | 6·0 |
| | | | 10.00 | 110 | 11·7 | 12·8 | 6·4 |
| | | | 11.30 | 120 | 13·3 | 16·0 | 10·0 |
| | | | Total for 2nd 12 hours | **530** | — | **78·8** | — |
| | | | Total for 24 hours | **1175** | — | **197·1** | — |
| 10th | 11.45 a.m. | 150 | 1.15 p.m. | 150 | 11·7 | 17·5 | 10·0 |
| | | | 2.30 | 130 | 21·7 | 28·0 | 22·4 |
| | | | 4.30 | 130 | 30·0 | 39·0 | 20·0 |
| | | | 11.45 | 500 | 7·5 | 37·5 | 5·0 |
| | | | Total for 1st 12 hours | **910** | — | **122·0** | — |
| | 11.45 p.m. | 150 | 8.00 a.m. | 480 | 15·0 | 72·0 | 8·7 |
| | | | 10.00 | 110 | 11·7 | 12·8 | 6·4 |
| | | | 11.30 | 120 | 11·7 | 14·0 | 10·0 |
| | | | Total for 2nd 12 hours | **710** | — | **98·8** | — |
| | | | Total for 24 hours | **1620** | — | **220·8** | — |
| 11th | 12.00 noon | 150 | Total for 1st 12 hours | **670** | **16·7** | **111·2** | **9·0** |
| | 12.00 midnight | 150 | Total for 2nd 12 hours | **630** | **16·7** | **105·0** | **8·7** |
| | | | Total for 24 hours | **1300** | — | **215·2** | — |
| 12th | 12.00 noon | 150 | Total for 1st 12 hours | **770** | **15·8** | **122·0** | **10·4** |
| | 12.00 midnight | 150 | 8.00 a.m. | 750 | 10·0 | 75·0 | 9·0 |
| | | | 10.00 | 100 | 10·0 | 10·0 | 5·0 |
| | | | 11.00 | 70 | 11·7 | 8·0 | 8·0 |
| | | | 12.00 noon | 80 | 8·3 | 5·3 | 5·3 |
| | | | Total for 2nd 12 hours | **1000** | — | **98·3** | — |
| | | | Total for 24 hours | **1770** | — | **220·3** | — |

oxidation with hydrogen peroxide *per se* is slow and troublesome, a more convenient method, which is incidentally capable of very broad application, was therefore developed. A small quantity of iodine was used as the oxidising agent and this was regenerated from the hydriodic acid formed by the action of hydrogen peroxide, thus making it available for further oxidation. In the presence of excess of hydrogen peroxide this cycle of reaction is continuous. The following are details of the procedure used in the experiment. To the concentrated decitrated lemon juice, brought to $p_H$ 3 with sulphuric acid, were added 1/50 of the amount of $N/10$ iodine necessary to oxidise the ascorbic acid and 1·5 times the theoretical quantity of $N$ hydrogen peroxide. The mixture was allowed to stand at room temperature until the indophenol-reducing capacity of the juice had just disappeared. The doses used in these experiments took 1–1·5 hours to become oxidised.

S. S. Z., who was "saturated" with ascorbic acid, placed himself on a scorbutic diet as described above. The first day he took a dose of 150 mg. of ascorbic

Table VIII.

| | Intake | | Excretion in urine | | | | |
|---|---|---|---|---|---|---|---|
| Day | Time dose taken | Dose mg. | Time urine passed | Volume of urine ml. | Ascorbic acid per 100 ml. urine mg. | Total amount of ascorbic acid mg. | Ascorbic acid output per hour mg. |
| 1st | 10.00 a.m. | 150 Ascorbic acid | 11.00 a.m. | 235 | 3·3 | 7·8 | 3·9 |
| | | | 1.00 p.m. | 300 | 6·7 | 20·0 | 10·0 |
| | | | 2.00 | 370 | 4·1 | 15·4 | 15·4 |
| | | | 4.00 | 140 | 6·7 | 9·3 | 4·7 |
| | | | 5.00 | 85 | 6·7 | 5·7 | 5·7 |
| | | | 10.00 | 230 | 5·5 | 12·7 | 2·5 |
| | | | 8.00 a.m. | 1100 | 1·3 | 13·8 | 1·4 |
| | | | 10.00 | 280 | 2·0 | 5·6 | 2·8 |
| | | | 12.30 p.m. | 305 | 2·1 | 6·6 | 2·6 |
| | | | Total | **3045** | — | **96·9** | — |
| 2nd | 12.00 noon | 250 Dehydro-ascorbic acid | 2.00 p.m. | 270 | 13·1 | 35·5 | 23·7 |
| | | | 3.00 | 170 | 10·0 | 17·0 | 17·0 |
| | | | 4.00 | 160 | 6·5 | 10·4 | 10·4 |
| | | | 5.00 | 300 | 4·0 | 12·0 | 12·0 |
| | | | 10.00 | 265 | 8·3 | 22·0 | 4·4 |
| | | | 8.00 a.m. | 750 | 1·8 | 13·8 | 1·4 |
| | | | 10.00 | 250 | 2·7 | 6·7 | 3·9 |
| | | | 12.00 | 190 | 2·5 | 4·8 | 2·4 |
| | | | Total | **2355** | — | **122·2** | — |
| 3rd | 12.30 p.m. | 220 Dehydro-ascorbic acid | 2.00 p.m. | 210 | 17·0 | 35·7 | 17·9 |
| | | | 3.00 | 160 | 15·8 | 25·3 | 25·3 |
| | | | 4.00 | 520 | 3·1 | 16·4 | 16·4 |
| | | | 5.00 | 380 | 3·7 | 13·9 | 13·9 |
| | | | 10.00 | 260 | 9·3 | 24·3 | 4·9 |
| | | | 8.00 a.m. | 860 | 3·1 | 27·2 | 2·7 |
| | | | 10.00 | 360 | 3·1 | 11·1 | 5·6 |
| | | | 12.00 | 160 | 4·3 | 6·9 | 3·5 |
| | | | Total | **2910** | — | **160·8** | — |
| 4th | 12.30 p.m. | | 2.00 p.m. | 280 | 3·3 | 9·3 | 4·7 |
| | | | 4.00 | 225 | 2·7 | 6·0 | 3·0 |
| | | | 5.00 | 120 | 2·8 | 3·4 | 3·4 |
| | | | 10.00 | 210 | 3·8 | 8·0 | 1·6 |
| | | | 8.00 a.m. | 630 | 2·0 | 12·6 | 1·3 |
| | | | 10.00 | 310 | 1·25 | 3·9 | 2·0 |
| | | | 12.00 | 225 | 1·3 | 3·0 | 1·5 |
| | | | Total | **2000** | — | **46·2** | — |

acid, on the second and third days 250 and 220 mg. of dehydroascorbic acid were ingested respectively. No dose was taken on the fourth day. The excretion during the first 24 hours of 90 mg. of the 150 mg. of ascorbic acid ingested on the first day (Table VIII) shows that the subject was "saturated." On the second day, of the 250 mg. of dehydroascorbic acid ingested, 122 mg. were excreted in the reduced form and similarly on the third day an even higher proportion was excreted in the urine as ascorbic acid. On the fourth day, when no dose was taken, the total amount of ascorbic acid in the urine as well as the hourly excretion fell to a low level. Similar results which are given in an abridged form in Table IX were obtained with S. W. J. and A. E. K. on diets very low in vitamin C.

Table IX.

| | | | Excretion in urine | | | | |
|---|---|---|---|---|---|---|---|
| Subject | Day | Intake mg. | Volume of urine ml. | Total ascorbic acid mg. | Maximum output of ascorbic acid per hour mg. | Time taken hours | Minimum output of ascorbic acid per hour mg. |
| S. W. J. | 1st | 300 Ascorbic acid | 1770 | 220·3 | — | — | 5·0 |
| | 2nd | 360 Dehydroascorbic acid | 1285 | 289·4 | 55·7 | 2·75 | 3·0 |
| A. E. K. | 1st | 150 Ascorbic acid | 990 | 96·0 | — | — | — |
| | 2nd | 370 Dehydroascorbic acid | 740 | 170·0 | — | — | 2·0 |
| | 3rd | 320 Dehydroascorbic acid | 950 | 205·7 | 34·3 | 3·5 | 1·1 |
| | 4th | — | 1425 | 22·0 | — | — | 0·6 |

The indophenol-reducing capacity of several specimens of urine from the above experiments could not be raised by treatment with hydrogen sulphide, showing that no dehydroascorbic acid was present in the urine.

In these experiments also it was found by a biological test that the indophenol-reducing capacity of the urine was mainly due to the presence of ascorbic acid.

## Discussion.

At the outset we are faced with the fact that there is no standard of normality as regards the urinary excretion of ascorbic acid by man. At one end of the scale are S. W. J., A. E. K., H. H. and the cases described by Hess and Benjamin, whose urines did not reduce indophenol to any significant extent for a number of days, even when "adequate" amounts of vitamin C were consumed daily. At the other end of the scale there is S. S. Z., who, because his intake of the vitamin was rather high, excreted in the urine as much as 70–150 mg. per day. The cases mentioned by Harris *et al.* apparently occupy an intermediate position. It is of course impossible at this stage of the inquiry to judge which of the three categories is the most prevalent, but that undoubtedly will depend on many factors, such as season of the year, individual habits, availability of antiscorbutic foods, *etc.*

The experiments show definitely that the store of ascorbic acid in the subject plays an important part in its urinary excretion. This fact also emerges from

Hess and Benjamin's work on children. Our results demonstrate that at least two factors are concerned with the appearance of ascorbic acid in the urine of normal individuals, the degree of "saturation" of the subject and the magnitude of the intake. When the store is low many days may elapse before a constant level of excretion, indicating "saturation," is reached if doses of the order of 150 mg. are taken. The time necessary to reach this condition can, however, be markedly shortened by ingestion of high doses (1000 mg.).

It is a striking fact that on discontinuing the dose when the subject is fully "saturated," the daily urinary output of ascorbic acid falls rapidly, reaching the initial low level in a little more than 24 hours. During this period of low output, the subject, however, remains "saturated" for some time. This fact, taken in conjunction with the observation that part of the ascorbic acid dose can be excreted in the urine even long before "saturation" is reached, makes it difficult to judge the degree of "saturation" of the subject from his level of urinary output taken at random. For example, S. W. J., who took daily about 120 mg. and excreted about 30 mg. (Table I) on each of several days, was not "saturated" since he did not reach his constant level of output until some time later. On the other hand, on discontinuing the dose a little later his urinary output fell within a few days even to a lower level, 6 mg. per day, yet he was "saturated" at the time, as is seen from the fact that a single dose of 180 mg. brought his output back to that of the "saturation" level (Table II). Further, when high doses are taken by an "unsaturated" subject, the daily urinary output may be very high while the subject is still in this condition. Such a case is illustrated by A. E. K. who, while still "unsaturated," returned 77 and 303 mg. on the second and third days respectively of the experiment (Table IV).

It must be particularly noted here that S. W. J., A. E. K. and H. H. existed for months on diets containing quantities of vitamin C which were sufficient to keep them in perfect health and apparently quite free from scurvy, yet their store of ascorbic acid was such that it took a number of days to bring about a condition of "saturation" on a daily dose of about 150 mg.

The results obtained with dehydroascorbic acid were so clear cut that they leave little room for comment.

The fate of the large quantities of ascorbic acid not accounted for in the urine when massive doses were ingested calls for further inquiry. At present it may be asserted almost with certainty that none of it was excreted in the urine as dehydroascorbic acid.

The exploratory experiments performed with the object of ascertaining whether under certain conditions such as complete "saturation" and a suitable dose, the balance between the intake and the urinary excretion of ascorbic acid could possibly be utilised as a means of determining the daily requirements of the vitamin were disappointing. Besides the experiments described, results obtained in this connection with other subjects (A. E. K. and H. H.) were equally unsatisfactory. It must, however, be pointed out that a final judgment may only be formed from work carried out on a greater number of subjects than we were able to employ.

During the process of this investigation we studied the effect of diuresis, induced by a high intake of water, on the urinary excretion of ascorbic acid. We found that it did not influence either the rate or the level of output. Although the quantities of liquid consumed are not given here, this fact can be appreciated by comparing the amounts of the ascorbic acid excreted with the corresponding volumes of urine passed in the various experiments.

Another point worthy of comment, which emerges from all the experiments described, is that although the rate of urinary excretion of ascorbic acid during the night was low it invariably increased soon after rising (period 8 a.m. to 10 a.m.).

## Summary.

The urinary excretion of ascorbic acid under normal conditions of existence is variable.

The output of ascorbic acid in the urine is conditioned by the amount stored in the body and by the quantity consumed in the diet.

When the store of ascorbic acid is complete, a more or less constant level of urinary excretion can be achieved, and this level varies with the magnitude of the daily quantity of ascorbic acid taken.

The percentage of ingested ascorbic acid excreted in the urine is lower when high doses are taken than when the doses are low. The actual amount of ascorbic acid not accounted for in the urine is therefore very much higher in the first than in the second case and is evidently not indicative of the real vitamin requirements of the individual. No dehydroascorbic acid is found in the urine when high doses are used.

On discontinuing the intake of ascorbic acid, a subject who is "saturated" may yet remain in this condition for some time despite the fact that his urinary excretion drops to a very low level.

When the subject is "saturated" or approaching "saturation" and a dose is taken, the rate of urinary excretion rapidly rises to a maximum in about 4 hours and then gradually returns to its initial rate in about 24 hours.

Diuresis induced by excessive intake of water does not influence either the rate or level of urinary output of ascorbic acid.

The rate of urinary excretion of ascorbic acid is low during the night and increases soon after rising.

When dehydroascorbic acid is ingested it appears in the urine as ascorbic acid and is excreted in the same way as the latter.

It is possible to exist on diets containing sufficient vitamin C to insure good health and freedom from scurvy even when the store of ascorbic acid in the body is maintained low.

It is shown by means of a biological test that the reducing capacity of the urine for indophenol in our experiments is due to ascorbic acid.

We wish to express out thanks to Mr A. E. Kellie and Mr H. Hutchins for kindly placing themselves at our disposal for experimental purposes. One of us (S. W. J.) is indebted to the Medical Research Council for a whole time grant.

## REFERENCES.

Euler and Klussman (1933). *Ark. Kem. Min. Geol.* **11** B, No. 7, 1.
Harris, Ray and Ward (1933). *Biochem. J.* **27**, 2011.
Hess and Benjamin (1934). *Proc. Soc. Exp. Biol. Med.* **31**, 855.
Van Eekelen, Emmerie, Josephy and Wolff (1933). *Nature*, **132**, 315.
Van der Walle (1922). *Biochem. J.* **16**, 713.

# CLXXXVIII. STUDIES IN TISSUE METABOLISM.
## IV. A COMPARISON OF MUSCLE AND TUMOUR GLYCOLYSIS.

By ERIC BOYLAND and COLIN ASHLEY MAWSON[1].

*From the Research Institute of the Cancer Hospital (Free), S.W. 3.*

(*Received June 22nd, 1934.*)

Since the work of Warburg [1930] on tumour metabolism it has been known that malignant tissue will produce lactic acid at a relatively high rate under either aerobic or anaerobic conditions. Warburg stated that tumour tissues can produce an amount of lactic acid equal to their own weight in ten hours, but this statement has been questioned by Downes [1929]. Chemical estimations indicate that slices of Jensen rat sarcoma will produce about 10 to 15 mg. lactic acid per g. (wet weight) per hour under anaerobic conditions at 37°. Owing to the rapid deterioration of the glycolytic power of minced muscle at this temperature, comparison is difficult, but Boyland [1931] found that minced ox-muscle at 20° formed 3 to 4 mg. lactic acid per g. of tissue per hour.

The mechanism of lactic acid formation in muscle resembles, according to Meyerhof [1930; 1933], the alcoholic fermentation of yeast in the fact that phosphorus compounds form an essential step in the breakdown of the sugar, and he places the glycolysis of erythrocytes in the same class [Meyerhof, 1932]. When materials so dissimilar as yeast, erythrocytes and muscle have similar glycolytic systems, it might be expected that tumour would behave in the same way, but to test this satisfactorily is not an easy matter. Owing to the limited permeability to phosphorus compounds of cell membranes [*cf.* Eggleton, 1933] it is essential that cell-free extracts, or some other non-cellular preparation, should be used.

Barr *et al.* [1928] found that the glycolytic activity of tumour tissue was very easily destroyed by any form of manipulation such as grinding, freezing or extraction. They conclude that there is no evidence for the formation of carbohydrate-phosphate compounds in cancer, and in this opinion they are supported by Harrison and Mellanby [1930]. These authors consider that tumour glycolysis differs fundamentally from that of non-malignant tissues in that it does not involve the reaction of phosphorus compounds.

Downes [1929] found that rat tumours would produce lactic acid from hexose-phosphate at about half the rate of glycolysis of glucose, and an indication that phosphate was at any rate to some extent involved in tumour metabolism was given by Franks [1932], who showed that the increase in free phosphate observed on incubation of mouse tumour C 180 was considerably reduced in the presence of glucose, while the acid-soluble organic phosphate at the same time increased. However, about twenty molecules of lactic acid were formed for each molecule of phosphate esterified, while under similar conditions in muscle three molecules of lactic acid were formed for each molecule of hexose ester produced.

[1] Halley Stewart Research Fellow.

The presence of hexosephosphate and adenylpyrophosphate in tumour tissue was indicated by the work of Boyland [1932] and Franks [1932], and this suggests that the glycolysis of muscle and tumour are similar. Tumour glycolysis, like that of muscle, is arrested by pancreatic extract, fluoride and iodoacetate. It is known that these substances interfere with the phosphate metabolism.

*Glycolytic enzymes from rat muscle.*

In order to compare the enzymes of muscle and tumour it was advisable to obtain these tissues from the same animal. Meyerhof [1930] has described the preparation of actively glycolysing cell-free extracts from the muscle of rabbits and frogs (in which experimental tumours were not available), but states that such extracts cannot be prepared from rat-muscle. We found, however, that the following procedure gave active extracts from rat-muscle.

Freshly excised muscle was rapidly chopped with scissors in its own weight of ice-cold water contained in a mortar cooled to about $-12°$. After about five minutes' rapid chopping one-tenth of the muscle's weight of sand was added and the whole thoroughly pounded until, after about ten minutes, it had solidified to a pasty mass. This material was centrifuged until it had completely thawed (usually about 15 minutes) and the clear supernatant liquid poured off through muslin. Occasionally a thixotropic gel was formed at this stage, but such gels were quite as active as the liquid extracts. After an experiment with such an extract the proteins were precipitated with 4 % trichloroacetic acid and lactic acid was estimated by the method described by Lohmann [1928].

Extracts from rat-muscle formed lactic acid from glycogen, hexosephosphate and α-glycerophosphate + pyruvate. (We are indebted to Dr F. L. Pyman for the pure sodium α-glycerophosphate.) Dialysis destroyed their glycolytic activity, which was restored on addition of adenylpyrophosphate and magnesium. No lactic acid was obtained from β-glycerophosphate in any circumstances. Results of experiments using rat-muscle extracts are given in Table I. The optimum concentrations of both phosphate and hydrogen ions for the formation of lactic

Table I. *Lactic acid formation in rat-muscle extract.*

(1) Dialysed extract.

| Substrate | Glycogen | Glycogen + adenylpyrophosphate + $MgCl_2$ | Hexosephosphate | Hexosephosphate + adenylpyrophosphate + $MgCl_2$ |
|---|---|---|---|---|
| mg. lactic acid formed in 2 hours by 2 ml. extract | 0·15 | 3·46 | 0·19 | 1·35 |

(2) Undialysed extract.

| Substrate | Glycogen | Pyruvate | α-Glycerophosphate | Pyruvate + α-glycerophosphate |
|---|---|---|---|---|
| mg. lactic acid formed in 2 hours by 2 ml. extract | 4·59 | 0·68 | 1·19 | 4·13 |

Table II. *Optimum* $p_H$ *of lactic acid formation in rat-muscle extract.*

1·5 ml. extract, incubated with glycogen, adenylpyrophosphate + Mg and phosphate buffer.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Exp. 1. | | | | | | | |
| $p_H$ of solution | 6·0 | 6·8 | 6·9 | 7·2 | 7·5 | 8·0 | |
| mg. lactic acid formed | 2·34 | 2·88 | 2·92 | 3·32 | 3·72 | 2·26 | |
| Exp. 2. | | | | | | | |
| $p_H$ of solution | 5·7 | 6·1 | 6·8 | 7·3 | 8·0 | 8·9 | 9·3 |
| mg. lactic acid formed | 0·37 | 0·46 | 1·23 | 1·77 | 1·76 | 0·96 | 0·23 |

acid from either glycogen or α-glycerophosphate + pyruvate were determined. The optimum $p_H$ was found to be about 7·5 (Table II) while at this $p_H$ the optimum concentration of phosphate was approximately 0·03 *M* (Table III). The hydrogen ion concentration was determined colorimetrically immediately after all additions had been made.

Table III. *Optimum phosphate concentration for lactic acid formation in rat-muscle extract at* $p_H$ *7·5.*

| | | | | | |
|---|---|---|---|---|---|
| Exp. 1. | | | | | |
| $PO_4$ concentration | *M*/3000 | *M*/1000 | *M*/300 | *M*/100 | *M*/30 |
| mg. lactic acid formed | 0·12 | 0·11 | 0·46 | 2·66 | 3·69 |
| Exp. 2. | | | | | |
| $PO_4$ concentration | *M*/64 | *M*/32 | *M*/16 | | |
| mg. lactic acid formed | 2·40 | 3·40 | 3·13 | | |

*Comparison of muscle and tumour glycolysis.*

A study was made of the effect of changing $p_H$ and phosphate concentration on tumour glycolysis. The presence or absence of phosphate in the medium had no effect on the glycolysis even if the tissue had been soaked in a large volume of oxygenated Ringer solution overnight and thoroughly washed next morning. This may be due to the fact that the tumour cell is impermeable to phosphate or contains large stores of available phosphate in the form of nucleic acids and phospholipins.

No sharply defined optimum $p_H$ could be determined for tumour tissue, but it appeared to be about $p_H$ 8 (Table IV).

Table IV. *Optimum* $p_H$ *for glycolysis of J.R.S. slices in phosphate and bicarbonate.*

| | | | | | |
|---|---|---|---|---|---|
| With bicarbonate buffer (*M*/50). | | | | | |
| $p_H$ | 8·9 | 8·4 | 7·6 | 7·1 | 6·5 |
| mg. lactic acid formed | 2·57 | 2·82 | 2·51 | 2·66 | 1·81 |
| With phosphate buffer (*M*/30). | | | | | |
| $p_H$ | 8·7 | 7·3 | 6·8 | 6·3 | 5·3 |
| mg. lactic acid formed | 2·38 | 2·38 | 1·78 | 0·92 | 0·49 |

Tumour slices formed lactic acid from hexosemonophosphate and from α-glycerophosphate + pyruvate, but to a smaller extent than from glucose (Table V). Addition of pyruvate did not appreciably increase the yield of lactic

Table V. *Glycolysis of J.R.S. slices with glucose, hexosemonophosphate and pyruvate + α-glycerophosphate, shaken in Warburg apparatus in presence of* $N_2 + 5\ \%\ CO_2$ *at 37°.*

| Substrate | Glucose | | Hexose-monophosphate | | Pyruvate + α-glycero-phosphate | |
|---|---|---|---|---|---|---|
| Time (mins.) | 45 | 90 | 45 | 90 | 45 | 90 |
| mg. lactic acid formed | 0·85 | 2·16 | 0·14 | 0·37 | 0·17 | 0·30 |

acid from hexosemonophosphate with either muscle extract or tumour slices. In order to investigate muscle and tumour under approximately similar conditions, comparisons of glycolytic power were made using the minced tissues. Results are given in Table VI, which show that an appreciable yield of lactic acid can be obtained from α-glycerophosphate + pyruvate with minced tumour, but this yield is not very much greater than that from pyruvate alone unless adenyl-

Table VI. *Glycolysis of minced tumour and rat-muscle, shaken in Warburg apparatus in presence of* $N_2+5\%\ CO_2$.

mg. lactic acid from 0·5 g. tissue + 3 ml. additions in two hours at 37°.

| | Substrate | | | | |
|---|---|---|---|---|---|
| Tissue | Pyruvate | Pyruvate + α-glycero-phosphate | Pyruvate + α-glycero-phosphate + adenylpyro-phosphate + Mg | Glucose (or glycogen with muscle) | Glucose + adenylpyro-phosphate + Mg |
| 1. J.R.S. | 0·33 | 0·53 | 0·63 | 2·45 | — |
| 2. J.R.S. | 0·40 | 0·68 | 0·97 | 3·19 | 3·04 |
| 3. J.R.S. | 0·65 | 0·99 | 1·09 | 2·79 | — |
| 4. L.R. 10 | 0·69 | 0·86 | 1·33 | 2·78 | 3·09 |
| 5. L.R. 10 | 0·34 | 0·62 | 0·99 | 2·82 | 3·06 |
| 6. Muscle | 0·47 | 1·62 | — | 3·35 | — |
| 7. Muscle | 0·47 | 2·15 | — | 5·16 | — |

The tumour L.R. 10 (no. 5 above) gave with hexosemonophosphate 0·30 mg. lactic acid, and with hexosemonophosphate + adenylpyrophosphate + Mg, 0·49 mg. L.R. 10 is a rat sarcoma, produced originally by injection of 1:2:5:6-dibenzanthracene [Burrows *et al.*, 1932].

pyrophosphate is added. If we consider the figures as a percentage of the maximum glycolysis (*i.e.* with glucose in the case of tumour and with glycogen in the case of muscle) it will be seen that the yield of lactic acid from tumour with α-glycerophosphate and pyruvate is comparable with the yield from muscle with the same compounds (Table VII).

Table VII. *Glycolysis of minced tumour and minced rat-muscle, shaken in Warburg apparatus in presence of* $N_2+5\%\ CO_2$.

Mean glycolysis as % of glycolysis with glucose (or glycogen for muscle).

| | Substrate | | |
|---|---|---|---|
| Tissue | Pyruvate | Pyruvate + α-glycerophosphate | Pyruvate + α-glycerophosphate + adenylpyro-phosphate + Mg |
| J.R.S. | 16 | 28 | 32 |
| L.R. 10 | 18 | 26 | 41 |
| Muscle | 11 | 44 | — |

Smythe and Gerischer [1933] have shown that the glyceraldehydephosphate which was synthesised by Fischer and Baer [1932] is rapidly fermented by yeast preparations. Through the generosity of Prof. H. Fischer of Basel and Dr Feldmann of Berlin, who gave us some calcium glyceraldehydephosphate, we have been able to observe the lactic acid formation from this substance by tumour slices and rat erythrocytes (Table VII). The amount of lactic acid formed in this way by tumour tissue is less than half that formed from glucose; it is therefore attacked at about the same rate as hexosephosphate or glycerophosphate + pyruvate. Rat erythrocytes also formed some lactic acid from glyceraldehydephosphate under the same conditions.

*Attempts to prepare cell-free extracts from tumours.*

Warburg [1930] and Barr *et al.* [1928] have attempted, without success, to obtain a glycolytic enzyme from tumour tissue, but as these experiments were done before the nature of the muscle coenzyme was known it was thought that

the addition of adenylpyrophosphate + Mg to tumour press juice might give an active cell-free extract: no success was attained by this method. While it was possible repeatedly to freeze rat-muscle extracts without loss of activity, tumour tissue suffered severe reduction in its glycolytic power if frozen once. Inactive extracts were obtained by grinding minced tumour or tumour slices with water, saline, boiled muscle extract, various antiprotease preparations, glucose solution, fluoride and alkaline phosphate solutions.

*Effect of haemolytic reagents.*

It seems probable that tumour does not attack glycogen (a large molecule), hexosephosphate and α-glycerophosphate (by analogy with sodium phosphate) because these substances do not penetrate the cell membrane, and so do not come into contact with the cell enzymes. In an attempt to increase the amount of diffusion into the cells various substances were added to tumour slices which should increase their permeability. Neither saponin, digitonin, taurocholate, oleate, ricinoleate nor choline appreciably increased the yield of lactic acid from α-glycerophosphate + pyruvate with tumour slices, though in some cases taurocholate and choline slightly increased the yield from glucose.

*Experiments with acetone slices.*

Harden and Young [Harden, 1932] were able to show the effect of phosphate on fermentation not only in yeast juice, which corresponds to muscle extract, but also in dried yeast and zymin. Meyerhof [1930] has described the preparation of an acetone precipitate from muscle extract having 40 % of the activity of the original extract. Using a similar precipitate from muscle extract, we obtained 1·91 mg. of lactic acid from glycogen and 1·74 mg. from α-glycerophosphate + pyruvate, whereas an equivalent amount of the original extract had given 5·63 mg. of lactic acid from glycogen. Analogous preparations were therefore made from tumour material.

Slices of good Jensen rat sarcoma (J.R.S.) tissue (0·25 mm. thick) were dried over $P_2O_5$, but had very little glycolytic activity even with the muscle coenzyme. Preparations with acetone, however, gave better results. Thin slices of J.R.S. or mouse sarcoma L.M. 154 were soaked in alkaline Ringer-bicarbonate solution ($p_H$ 8·7) for 15 minutes, the Ringer drained away and the slices dropped into about 100 volumes of acetone at 37°. The acetone was rapidly stirred and decanted after two minutes. A.R. acetone at 37° (dried over anhydrous $Na_2SO_4$) was then added and left at this temperature for 15 minutes. After pouring off the acetone, the slices were rapidly drained and dried overnight *in vacuo* over $CaCl_2$. The dried material (9–12 % of the original wet weight) was white and became swollen in water so as to resemble the original tissue. Although such preparations were sometimes inactive, acetone slices have been obtained having up to about one-tenth of the glycolytic activity of the original tissue.

0·3 g. of dried acetone J.R.S. slices was soaked in 3 ml. rat Ringer solution and finely minced. 0·3 ml. of the emulsion so formed was injected into each of ten rats. In no case was there any growth of tumour tissue even after 3 months.

The acetone slices contained some lactic acid precursor, for on incubation alone some lactic acid was usually obtained, but an increased amount was produced on incubation with glucose or pyruvate + α-glycerophosphate. If the preparation was washed with $M/100$ phosphate buffer ($p_H$ 7·4) containing toluene, and then repeatedly with toluene water, the lactic acid precursor could be removed.

Table VIII. *Lactic acid formation from glyceraldehydephosphate by tumour and erythrocytes.*

Lactic acid formed in 2 hours while shaking at 37° in Warburg apparatus.

| | Substrate | mg. lactic acid |
|---|---|---|
| 100 mg. J.R.S. | None | 0·10 |
| | Glucose (4 mg.) | 1·25 |
| | Calcium glyceraldehydephosphate (4 mg.) | 0·53 |
| 20 mg. Rat erythrocytes | None | 0·02 |
| | Calcium glyceraldehydephosphate (4 mg.) | 0·36 |
| | Theoretical lactic acid formation from 4 mg. calcium glyceraldehydephosphate | 1·48 |

Table IX. *Lactic acid formation by acetone-treated tumour slices incubated in $N_2+5\%$ $CO_2$ at 37°.*

mg. lactic acid per 100 mg. dry weight per hour.

| | | | Substrate | | | |
|---|---|---|---|---|---|---|
| Exp. | Tumour | Incubation time (mins.) | Glucose | Glucose + adenylpyrophosphate + $MgCl_2$ | Sodium glycerophosphate + pyruvate | Sodium glycerophosphate + pyruvate + adenylpyrophosphate + $MgCl_2$ |
| 1 | L.M. 154* | 90 | 0·47 | 0·62 | 0·42 | — |
| 2 | J.R.S. | 150 | 0·35 | 0·34 | — | — |
| 3 | J.R.S. | 180 | 0·49 | 0·61 | 0·10 | 0·12 |
| 4 | J.R.S. | 180 | 0·53 | 0·55 | 0·15 | 0·64 |
| 5 | J.R.S. | 180 | 0·05 | 0·32 | 0·03 | 0·02 |
| 6 | J.R.S. | 180 | — | 0·13 | — | 0·09 |
| 7 | L.M. 154 | 180 | — | 0·53 | — | 0·28 |
| 8 | J.R.S. | 180 | 0·18 | 0·36 | 0·36 | 0·42 |
| 9 | J.R.S. | 180 | 0·07 | 0·13 | 0·33 | 0·36 |
| 10 | J.R.S. | 15 | 1·05 | — | 0·43 | — |
| | | 30 | 0·86 | — | 0·28 | — |
| | | 45 | 0·62 | — | 0·23 | — |
| 11 | J.R.S. | 15 | — | 0·64 | — | 0·48 |
| | | 30 | — | 0·58 | — | 0·56 |
| | | 60 | 0·29 | 0·50 | 0·38 | 0·44 |
| 12 | J.R.S. | 15 | — | 1·08 | — | 0·20 |
| | | 33 | — | 0·68 | — | 0·22 |
| | | 56 | 0·85 | 0·73 | 0·19 | 0·23 |
| 13 | J.R.S.† | 30 | 0·11 | — | 0·08 | 0·13 |
| | washed | 70 | 0·10 | 0·07 | 0·10 | 0·13 |
| 14 | J.R.S. | 45 | — | 0·30 | — | 0·36 |
| | | 90 | — | 0·23 | — | 0·23 |
| 15 | J.R.S. | 45 | — | 0·85 | — | 0·90 |
| 16 | J.R.S. | 45 | — | 0·56 | — | 0·40 |
| | | 90 | — | 0·36 | — | 0·32 |
| 17 | J.R.S.‡ | 60 | — | 0·20 | — | 0·19 |
| | washed | 120 | 0·09 | 0·52 | — | 0·34 |

* L.M. 154 refers to a grafted tumour produced in mice by the injection of 5:6-*cyclo*-penteno-1:2-benzanthracene [Barry and Cook, 1934].

† The media used for this experiment contained toluene and gave a vigorous growth of *B. coli* on incubation with broth containing toluene.

‡ Samples of the media used were taken at the end of incubation and found to be sterile.

The lactic acid formation of this non-living preparation of tumour tissue was small compared with that of the original tissue. In order to show that this glycolysis was not due to bacterial contamination, experiments under sterile conditions were required. Even after taking great precautions to ensure sterility by aseptic methods alone, incubation of the media at the end of the experiments showed that bacteria were present. The bacteria did not appear to be affected by saturation with thymol or with toluene. Eventually sterility was attained by working under aseptic conditions and adding 0·1 % phenol to the media. The sterile experiment (17 of Table IX) which was carried out on washed material gave a larger yield of lactic acid from glucose in the presence of coenzyme than did many experiments which showed contamination with either *Staphylococcus albus* or *B. coli*.

The foregoing experiments indicate the difficulty of obtaining a cell-free glycolysing extract which could be compared with muscle extract. The most active of the acetone slice preparations had less than one-tenth of the original activity. This remaining activity involves the same chemical mechanism as that of muscle. It may be dangerous to argue from this to the total glycolysis, since Ashford [1933] has suggested that glucose breakdown in brain may follow two paths, one involving phosphorylation and the other not. However, no glycolysis without phosphorylation has yet been clearly demonstrated. It is also possible that some of the residual activity remaining after treatment with acetone is due to red blood corpuscles which remain in the tissue.

## Summary.

1. Actively glycolysing extracts have been prepared from rat-muscle which give lactic acid with glycogen, hexosemonophosphate and α-glycerophosphate + pyruvate.

2. The optimum $p_H$ for glycolysis in rat-muscle extract is about 7·5, and the optimum phosphate concentration about $M/30$.

3. The glycolysis of tumour tissue is unaffected by the presence or absence of phosphate. The optimum $p_H$ is about 8·0.

4. A little lactic acid was formed from hexosemonophosphate and from α-glycerophosphate + pyruvate by tumour slices, but the amount is very much smaller than that formed by muscle extract from the same substances.

5. It was not found possible to obtain glycolytically active extracts from tumour tissue or to increase its metabolism of phosphorus compounds by the addition of haemolytic reagents.

6. A preparation made from tumour slices with acetone is described which possesses glycolytic activity; the yield of lactic acid from α-glycerophosphate + pyruvate is of the same order as that from glucose.

We are indebted to Dr C. C. Bryson, Assistant Pathologist to the Cancer Hospital for the care with which he examined for micro-organisms the numerous samples submitted to him, and to Mrs Boyland for manipulation of the Warburg apparatus.

REFERENCES.

Ashford (1933). *Biochem. J.* **27**, 903.
Barr, Ronzoni and Glaser (1928). *J. Biol. Chem.* **80**, 331.
Barry and Cook (1934). *Amer. J. Cancer*, **20**, 58.
Boyland (1931). *Biochem. Z.* **237**, 418.
—— (1932). *J. Physiol.* **75**, 136.
Burrows, Hieger and Kennaway (1932). *Amer. J. Cancer*, **16**, 57.
Downes (1929). *J. Cancer Research*, **13**, 268.
Eggleton (1933). *J. Physiol.* **79**, 31.
Fischer and Baer (1932). *Ber. deutsch. chem. Ges.* **65**, 337, 1040.
Franks (1932). *J. Physiol.* **74**, 195.
Harden (1932). Alcoholic fermentation. (Longmans Green and Co., London.)
Harrison and Mellanby (1930). *Biochem. J.* **24**, 141.
Lohmann (1928). Die Methodik der Fermente. Oppenheimer-Pincussen, **3**, 1236. (Thieme, Leipzig.)
Meyerhof (1930). Die Chemischen Vorgänge in Muskel. (Springer, Berlin.)
—— (1932). *Biochem. Z.* **246**, 249.
—— (1933). *Nature*, **132**, 337.
Smythe and Gerischer (1933). *Biochem. Z.* **260**, 414.
Warburg (1930). The metabolism of tumours. (Translated by F. Dickens. Constable, London.)

# CLXXXIX. STUDIES IN TISSUE METABOLISM.
## V. THE LACTIC DEHYDROGENASES OF YEAST AND HEART-MUSCLE.

BY ERIC BOYLAND AND MARGARET ESTHER BOYLAND.
*From the Research Institute of the Cancer Hospital (Free), S.W. 3.*

(*Received June 22nd, 1934.*)

STEPHENSON [1928] and Bernheim [1928] obtained lactic dehydrogenase of *B. coli* and of baker's yeast respectively in solution. Both preparations oxidised lactic acid to pyruvic acid and did not appear to have any coenzyme. Bernheim dialysed his preparations for 7 hours without destroying the activity. One of us [Boyland, E., 1933, 2] found that zymin from baker's yeast could be washed with large quantities of water without loss of lactic dehydrogenase activity [see also Harden and Macfarlane, 1931]. Within the last few years the lactic dehydrogenase of mammalian heart-muscle has been investigated by Szent-Györgyi [1925], who has shown that this enzyme requires a coenzyme, the nature of which he has attempted to determine [Banga *et al.* 1932; Banga and Szent-Györgyi, 1933]. The coenzyme is not adenylpyrophosphate [Banga and Szent-Györgyi, 1932] or vitamin $B_1$ [Boyland, E., 1933, 1; Birch and Mann, 1934], nor is it identical with glucose dehydrogenase coenzyme of liver (Harrison, personal communication, and observations with a sample kindly supplied by Dr Harrison). Holmberg [1934] found that cozymase or Fiske's adenylpyrophosphate could act as coenzyme for lactate oxidation by heart-muscle in the presence of methylene blue.

*Preparation of heart-muscle dehydrogenase in solution.*

Szent-Györgyi has always worked with the heart-muscle enzyme in suspension in the form of washed minced muscle, but the enzyme may be obtained in solution. Holmberg [1934] and Birch and Mann [1934] have prepared soluble enzymes from fresh mammalian muscle, Holmberg by extraction of horse skeletal muscle with bicarbonate; Birch and Mann from pig's heart by extraction with alkaline phosphate.

In the present work sheep or bullock hearts fresh from the slaughter-house were freed from fat, finely minced and ground with two volumes of acetone. The acetone was filtered off and the residue treated with another two volumes of acetone, filtered and then dried in a thin layer on filter-paper. The dried heart-muscle obtained in this way was stable for months and after washing with water gave a lactic dehydrogenase preparation as active as the original heart-muscle washed with water. To obtain an extract 15 g. of the acetone-dried heart-muscle are washed with 3 portions of 250 ml. water containing 2 or 3 drops of toluene. The washed material is then suspended in 50 ml. $M/15$ $Na_2HPO_4$ at 20°. The solid is removed by centrifuging, and the extract containing the enzyme may be clarified by filtration through Kieselguhr. The enzyme can be precipitated by saturation with ammonium sulphate. The extract does not reduce

methylene blue either alone or in the presence of the coenzyme alone (prepared according to Banga *et al.* [1932]) or of lactate alone. With lactate and coenzyme it rapidly reduces methylene blue. The activity of the coenzyme was found to decrease slowly over a period of weeks, even when kept in a desiccator.

The yeast lactic dehydrogenase was made by Bernheim's method [1928], except that the zymin from baker's yeast was well washed with water so as to obviate the necessity for dialysis of the final extract [Boyland, E., 1933, 2][1]. The yeast lactic dehydrogenase in solution is not so stable as that of heart-muscle and during the long dialysis necessary to remove substrates some deterioration of the enzyme must occur.

*The* $p_H$ *optimum of yeast and heart-muscle lactic dehydrogenase.*

The activity of the preparations was measured by the Thunberg technique. 1–3 ml. of enzyme solution (or 0·5 g. washed acetone heart-muscle) were mixed with *M*/15 buffer, *M* lithium lactate, and in the case of heart-muscle enzyme 0·5 to 1 mg. of coenzyme preparation in the Thunberg tube. 0·1 ml. of this mixture was taken for colorimetric estimation of the $p_H$ with a B.D.H. capillator. One drop of caprylic alcohol and 0·5 ml. 1/5000 methylene blue were added, and the tube was evacuated with a "Hyvac" pump until the liquid boiled briskly. The tube was then placed in a water-thermostat and the time taken for 90 % decoloration of the methylene blue determined. When the methylene blue was completely decolorised the tube was opened and the $p_H$ again rapidly determined. The $p_H$ values obtained in this way are probably as reliable as determinations with a quinhydrone or hydrogen electrode. A glass electrode was not available, but Dr Morton, of the Chelsea Polytechnic, kindly made determinations with this instrument of zymin suspensions and extracts at about the optimum $p_H$ and found an error of $+0{\cdot}2$ $p_H$ in each case. A correction of $0{\cdot}2$ $p_H$ has therefore been applied to the values determined colorimetrically for zymin.

Preliminary experiments had shown that heart-muscle lactic dehydrogenase was most active in alkaline solution. As sodium borate was found to inhibit the enzyme, borate buffers could not be used. From $p_H$ 4·0 to 9·5 a mixture of barbitone [Britton and Robinson, 1931] and phosphates was used. As a check glycine-phosphate-NaOH buffer was used and gave the same results. At $p_H$ 6·0 the heart-muscle enzyme was found to be inactive; from $p_H$ 9·3 to 9·5 the activity was at a maximum (Fig. 1). The relative activity was determined at 18°, 28° and 38° and the results are given in Table I, which also shows the temperature

Table I. *Effect of temperature on heart-muscle extract.*

| | Decoloration time (mins.) | | | $Q_{10}$ | |
|---|---|---|---|---|---|
| $p_H$ | 18° | 28° | 38° | 18–28° | 28–38° |
| 8·0 | — | 43 | 19 | — | 1·8 |
| 8·5 | 55 | 24 | 11 | 2·3 | 2·2 |
| 9·0 | 30 | 14 | 7·5 | 2·1 | 1·9 |

increment ($Q_{10}$). The relative activity of washed heart-muscle suspension was also determined at different hydrogen ion concentrations at 38°. The same result was obtained for this suspension as for the extract.

[1] We are indebted to the Distillers' Company for supplying the baker's yeast used throughout this work.

Barron and Hastings [1933] found the temperature increment of this enzyme from gonococci to be 2·23. Holmberg [1934] found that his enzyme was much more active at $p_H$ 8·0 than in more acid solution and his times of decoloration of methylene blue were

| $p_H$ | 6·2 | 6·6 | 7·0 | 7·5 | 8·0 |
|---|---|---|---|---|---|
| Time (mins.) | — | 32 | 18 | 6 | 4 |

He does not record results with a solution at a reaction more alkaline than $p_H$ 8·0.

The yeast enzyme was examined as a suspension of washed zymin from baker's yeast and in solution; the results are expressed in Fig. 1. Above $p_H$ 9·0, at which the heart-muscle enzyme was found to have a maximum, the yeast dehydrogenase was almost inactive. In phosphate buffer the suspension of

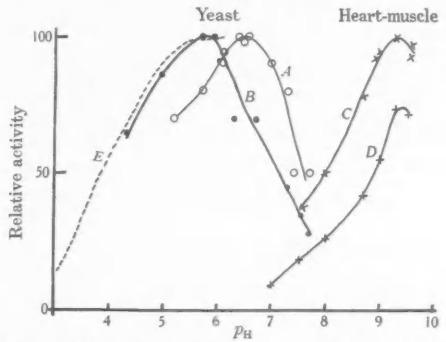



Fig. 1. Relative activities of lactic dehydrogenases of yeast and heart-muscle.

| | | |
|---|---|---|
| *A* | o | Zymin extract with lactate. |
| *B* | • | Zymin suspension with lactate. |
| *C* | × | Heart-muscle extract with lactate. |
| *D* | + | Heart-muscle extract with malate. |
| *E* | --- | Dissociation curve of lactic acid. |

washed zymin had a maximum activity at about $p_H$ 5·6, while the optimum $p_H$ for the extract was 6·2 (Fig. 1). This difference may be only apparent as it is possible that the enzyme of the washed zymin suspension is surrounded by protein so that the $p_H$ at the enzyme surface is different from that in the medium. The curve for the suspension approximates to the dissociation curve of lactic acid between $p_H$ 4 and 6.

Table II. *The effect of yeast extract on heart-muscle lactic dehydrogenase.*

| Preparation incubated | Time to reduce 1 ml. 1/10,000 methylene blue at 38° mins. |
|---|---|
| Washed zymin extract with *M*/10 lactate at $p_H$ 6·4 | 6 |
| ,, ,, at $p_H$ 9·3 | 36 |
| ,, ,, after boiling, at $p_H$ 6·4 | > 60 |
| Heart-muscle extract at $p_H$ with lactate | >100 |
| ,, ,, +1 mg. coenzyme | 7 |
| ,, ,, +1 ml. washed zymin extract | 36 |
| ,, ,, +1 ml. boiled washed zymin extract | > 60 |

*The absence of coenzyme from yeast lactic dehydrogenase.*

As the yeast enzyme was inactive at the $p_H$ at which that from heart-muscle showed maximum activity, the yeast extract could be tested for coenzyme by comparing its effect with that of the coenzyme preparation at $p_H$ 9·3. The results in Table II show that the yeast lactic dehydrogenase either fresh or inactivated by boiling had no coenzyme activity.

*The effect of heart-muscle lactic dehydrogenase on other substrates.*

The soluble lactic dehydrogenase of heart-muscle oxidised malate, but not succinate, pyruvate, tartronate, α-glycerophosphate or glucose. It was inhibited strongly by pyruvate and slightly by succinate (Table III).

Table III. *Effect of substrates on lactic dehydrogenase at 38°.*

| Heart-muscle extract incubated with | $p_H$ | Time taken to reduce 0·5 ml. 1/5000 methylene blue (mins.) Without coenzyme | With coenzyme |
|---|---|---|---|
| *M*/20 sodium succinate | 6·0 | >200 | >200 |
| *M*/20 sodium tartronate | 7·0 | >200 | >200 |
| *M*/20 sodium pyruvate | 8·0 | >200 | >200 |
| | 9·0 | >200 | >200 |
| *M*/20 sodium glycerophosphate | 7·2 | >200 | >200 |
| | 9·0 | >200 | About 200 |
| *M*/20 glue | 7·0 | >200 | ,, 200 |
| | 9·0 | >200 | ,, 150 |
| *M*/20 lactate | 8·8 | >200 | 10 |
| ,, + *M*/10 sodium succinate | 8·8 | — | 14 |
| ,, + *M*/750 sodium pyruvate | 9·0 | — | 28 |
| ,, + *M*/150 sodium pyruvate | 9·0 | — | 50 |
| ,, + *M*/30 sodium pyruvate | 9·0 | — | 85 |

The inhibitory action of pyruvate is similar to that found by Bernheim [1928] for zymin.

The effect of the heart-muscle extract on sodium malate was determined at different hydrogen ion concentrations as shown in Fig. 1. The optimum $p_H$ was about 9·3, which is the same as that found with lactate, but at $p_H$ 8·4 malate is acted on only at a third of the maximum rate, while at this $p_H$ lactate is oxidised at 70 % of the maximum rate. Even at the optimum $p_H$ malate is dehydrogenated at only about 50–70 % of the rate of lactate. This probably accounts for the fact that the oxidation of malate by heart-muscle has not been more often observed.

Table IV. *Relative rates of reduction of methylene blue by heart-muscle extract in the presence of lactate and malate at* $p_H$ *9·0 at 38°.*

| Extract incubated with | Relative activity |
|---|---|
| *M*/10 lactate + coenzyme | 100 |
| *M*/10 malate + coenzyme | 46 |
| *M*/5 malate + coenzyme | 51 |
| *M*/10 malate + *M*/10 lactate + coenzyme | 82 |

Holmberg [1934] found that his enzyme preparation partially decolorised methylene blue and completely decolorised thionin in the presence of *l*- and *dl*-malate. We have always observed complete decoloration of methylene blue, even at $p_H$ 7 when the reaction proceeds very slowly.

## Discussion.

The difference in the $p_H$ optima of the two enzymes is comparable with the difference in the $p_H$ optima of pepsin and trypsin, which both hydrolyse the peptide linkage. In the case of the peptide-splitting enzymes the $p_H$-activity curve and the titration curves of their protein substrates approximate to each other. In the case of yeast lactic dehydrogenase the activity increases as the lactic acid dissociation increases, and part of the $p_H$-activity curve of a washed zymin suspension coincides with the dissociation curve of lactic acid (see Fig. 1). This indicates that the enzyme acts upon charged lactate ions and not upon undissociated molecules.

As the heart-muscle enzyme is active in alkaline solution it must also act upon lactate ions. The $p_H$-activity curve of this enzyme cannot be explained by the dissociation of the carboxyl group, but it is possible that the hydroxyl group of lactate ions may change in the alkaline solutions in which heart lactic dehydrogenase operates.

Although lactic dehydrogenase reduces methylene blue in the presence of lactate but not in the presence of any other substrate (except malate with heart-muscle enzyme) it has not yet been possible to identify pyruvic acid as a product of the action of the enzyme.

## Summary.

1. The lactic dehydrogenases of mammalian heart-muscle and baker's yeast have been prepared in solution.

2. The optimum $p_H$ of the heart-muscle lactic dehydrogenase in solution is about 9·3; the lactic dehydrogenase of yeast has an optimum about $p_H$ 6·4.

3. The yeast enzyme is independent of coenzyme and does not attack malate, while the heart-muscle lactic dehydrogenase requires a coenzyme and oxidises both lactate and malate in alkaline solution.

One of us (M. E. B.) has pleasure in thanking the House Committee of the Cancer Hospital for a scholarship held during the progress of this work.

## REFERENCES.

Banga and Szent-Györgyi (1932). *Biochem. Z.* **247**, 216.
—— —— (1933). *Z. physiol. Chem.* **217**, 39.
—— —— and Vargha (1932). *Z. physiol. Chem.* **210**, 228.
Barron and Hastings (1933). *J. Biol. Chem.* **100**, 155.
Bernheim (1928). *Biochem. J.* **22**, 1178.
Birch and Mann (1934). *Biochem. J.* **28**, 622.
Boyland, E. (1933, 1). *Biochem. J.* **27**, 786.
—— (1933, 2). *Biochem. J.* **27**, 791.
Britton and Robinson (1931). *J. Chem. Soc.* 1456.
Harden and Macfarlane (1931). *Biochem. J.* **25**, 818.
Holmberg (1934). *Skand. Arch. Physiol.* **48**, 1.
Stephenson (1928). *Biochem. J.* **22**, 605.
Szent-Györgyi (1925). *Biochem. Z.* **157**, 50.

# CXC. THE EXUDATION OF GLUTAMINE FROM PERENNIAL RYE-GRASS.

By ALEC WALTER GREENHILL and ALBERT CHARLES CHIBNALL.

*From the Imperial Chemical Industries, Limited, Agricultural Research Station, Jealotts Hill, Bracknell, Berks, and the Biochemical Department, Imperial College, South Kensington.*

(*Received June 25th, 1934.*)

During the course of an investigation in which one of us (A. W. G.) was studying the effect of various levels of supply of nitrogen and of phosphorus, and the comparative effects of nitrate and of ammonia on the nitrogen and phosphorus nutrition of perennial rye-grass growing in pots, his attention was drawn unexpectedly one morning, March 19th, 1933, to a very striking phenomenon which had occurred overnight in the case of certain of the grasses undergoing treatment. The ends of the leaf blades of these particular grasses looked as if a whitewash brush had been drawn over them, and closer examination revealed the presence of a white substance adhering firmly to the ends.

The more detailed circumstances of the exudation were as follows. The grass had been established in the pots during the previous summer, in an artificial soil consisting of a mixture of sand with calcium bentonite and calcium carbonate, by feeding with suitable nutrient solutions coupled with very lenient cutting [Greenhill and Page, 1933]. The pots had been left untouched (except for watering when necessary) over the winter, with two to three inches of growth on them, and had been cut back to within one inch of soil level during the period March 13–16, before beginning the investigation proper. The grass at this time was making a noticeable amount of early spring growth. On March 17–18 the pots were given their respective phosphorus and nitrogen experimental treatments, which comprised three levels of phosphorus in all combinations with three levels of nitrogen, the latter being given in one series throughout as ammonium sulphate and in a second series as calcium nitrate. A uniform application of potassium sulphate was also given, while in the case of certain of the nitrogen and phosphorus treatments a second (lower) level of potash was included as well. All treatments were triplicated. The nutrients were all applied in solution by surface sprinkling, the phosphate and potash on March 17 and the nitrogen on March 18, and were followed finally on March 18 by a watering with distilled water to ensure that the salts were washed into the soil.

On the following morning, March 19, the exudation already described was observed in all those pots which had received ammonium sulphate at the highest rate of application, while it was present also, though to a much less extent, in the pots which had been treated at the intermediate rate. No exudation was present in the pots which had received the ammonium sulphate at the lowest rate, or in any of the pots which had received their nitrogen as calcium nitrate whether at high, intermediate or low rates. During the period March 19–22 the exudation

showed some increase in amount in the high and intermediate ammonium sulphate pots, while very slight traces appeared also in those with low ammonium sulphate and high calcium nitrate, but no trace was observable in the intermediate or low calcium nitrate pots. No further exudation occurred after March 22.

The exudation was confined to those leaves which had been recently cut, and further, to the immediate neighbourhood of the cut ends themselves; none was observed at other parts of the leaf, or at any part of the newly growing leaves which had suffered no defoliation. Also, although the grass was watered frequently and exposed to varying weather conditions, the exudation remained unchanged and firmly adherent to the leaf blades and presented a very striking appearance for nearly a fortnight, though subsequently it gradually disappeared. The extent of the exudation did not seem to be influenced by the differential phosphate or potash treatments, nor did the exudation appear to affect in any way the growth of the grass, which proceeded in every respect quite normally and satisfactorily. Fig. 1 (taken March 24) shows typical leaf blades with and without the exudation adhering.

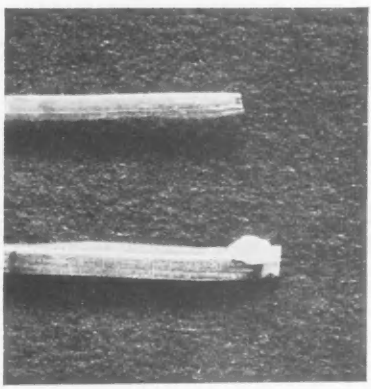


Fig. 1. J. H. 488. March 24, 1933.

Some of the exudation was removed from the leaves by means of a penknife blade on March 22 and 23 for chemical examination. The material was a white rather coarse powder which dissolved fairly readily in warm water, leaving a small residue of leaf fragments. The ash content was 10 %, and the total N by micro-Kjeldahl 14·4 %, or on an ash-free basis 16·2 %. 6·17 mg. were distilled *in vacuo* with a suspension of magnesia at 40° for 15 minutes, when ammonia equivalent to only 3·7 % of the total N passed over. The residue in the distillation flask was washed out and sufficient HCl added to bring the acid strength up to *N*. Boiling for 2 hours (Sachsse hydrolysis) yielded on distillation with excess of magnesia 48 % of the total N as ammonia.

The residue in the distillation flask was neutralised with glacial acetic acid and made up to standard volume. The amino-N determined by Van Slyke's micro-method was 52 % of the total N. These results showed clearly that the exudation consisted chiefly of either asparagine or glutamine. Proof that it was the latter substance was obtained by heating at $p_H$ 8 for 3 hours at 100°, which

split off 74 % of the total amide-N as ammonia [Chibnall and Westall, 1932]. Accordingly 60 mg. were dissolved in about 1 ml. of warm water, and an equal volume of warm alcohol was added. On standing typical needle-shaped crystals of glutamine separated. These were collected and recrystallised from aqueous alcohol. The material contained no water of crystallisation and gave no biuret reaction. (Found: C, 40·6; H, 6·8; N, 19·1 %. $C_5H_{10}O_3N_2$ requires C, 41·1; H, 6·9; N, 19·2 %.)

In the subsequent course of the experiment the grass in all pots was cut to within three-quarters of an inch of soil level in late April, after which the pots were again treated with nitrogen, phosphate and potash, and the plants cut back three or four weeks later. This alternating procedure of nutrient treatment and cutting after the grass had made three to four weeks' growth was repeated throughout the summer. Following each subsequent addition of the nutrient solution the exudation was again observed, but in no instances was it so striking as in the first one already described, and in no other was there sufficient material for a detailed chemical examination. The exudation on these subsequent occasions differed also from that described above in that it was not confined to the end of the cut blade, as on the first occasion, but appeared at various parts of the upper half of the blade, though always, as before, on a recently cut blade. Also, after the first two occasions, the exudation was confined almost entirely to the intermediate and low ammonia treatments.

In view of the foregoing observations in 1933, it was decided to study this spring (1934) the possible occurrence of glutamine in the leaves of perennial rye-grass actually growing in a natural pasture. Accordingly a small area of a pasture containing a high proportion of perennial rye-grass, which had been cut last

Fig. 2.

autumn but not cut or grazed since, was treated with ammonium sulphate at the rate of 2 cwt. per acre (a normal rate employed in agricultural practice) on March 23, and careful observation of the grass made daily. The perennial rye-grass at this time was making a little early spring growth. On March 26 a small amount of exudation (similar in appearance to that observed last year in the pots) was observed on a few of the leaf blades, including both those blades which had been cut last autumn and new ones which had grown since. The exudation was not confined to the end or tip of the blade, but was observed at various

points over the upper half; none was observed however on the lower half of the blade. A photograph of the exudation showing its presence down the side of newly-grown blades is reproduced in Fig. 2. An examination of the exudation in the laboratory showed it to contain no ammonium salt, but it was not possible to collect sufficient material for a more detailed chemical analysis.

It should be mentioned that the phenomenon this time (in the natural pasture) would not ordinarily have been observed by the passer-by, but it was sufficiently marked to be easily noticed on looking closely into the sward. On March 27 a sample of the upper parts of the blades was cut with a pair of scissors and examined. The grass was found to contain a high concentration of glutamine, equivalent to 32 % of the total water-soluble N. No further exudation from the grass occurred, and the material exuded disappeared within a few days.

It is perhaps worthy of mention that a similar exudation was observed on some of the pasture manuring experimental plots at Jealotts Hill following an application of ammonium sulphate at the rate of 2 cwt. per acre earlier in the month, while traces were observed also on similar plots receiving only 1 cwt. per acre, but none on plots receiving no nitrogen. In other plots, however, receiving nitrogen in different forms including ammonium sulphate at the rate of 1 cwt. per acre or its nitrogen equivalent, at the end of March, no signs of any exudation were observed.

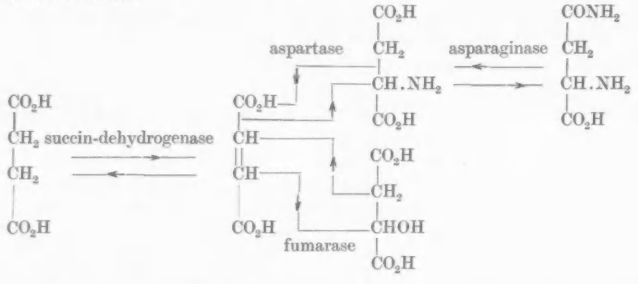
## Discussion.

This rapid elaboration of glutamine from ammonium salts raises an interesting new point in connection with the nitrogenous metabolism of the plant. As is well known, chiefly from the work of Schulze at Zürich, the growth of most seedlings, especially in the dark, results in a large accumulation of asparagine (up to 27 % of the total dry weight). In certain cases, especially in the Caryophyllaceae, glutamine replaces asparagine, but it is formed in much smaller amount (up to 5 % of the total dry weight), while very occasionally both amides are formed [*cf.* Stieger, 1913].

Schulze's results were confirmed by Prianischnikoff, who showed in addition that if seedlings were grown on solutions of ammonium salts the ammonia absorbed, if in excess of immediate protein requirements, was largely stored as asparagine. Probably on account of the greater difficulty in isolating and characterising glutamine, the storage of this amide under similar conditions has not hitherto been observed, and it is therefore of particular interest to note that such storage does actually take place in the case of perennial rye-grass. Since the exudation of glutamine is most noticeable in the early spring, when the absorption of ammonium salts by the root system is usually most active, it appears that the formation of this amide is a parallel case with that of asparagine, and that it is an innocuous form of storage for ammonia present in excess of immediate protein requirements.

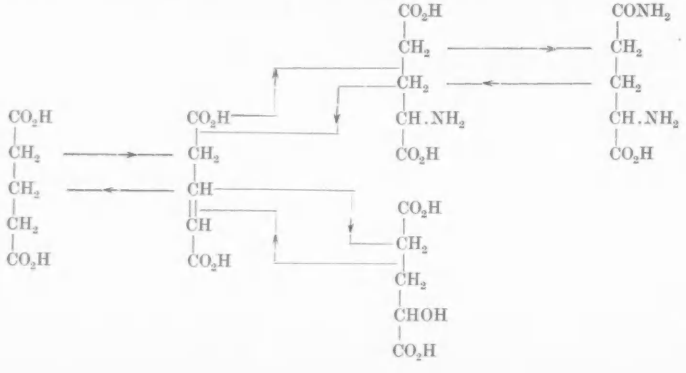
The exudation of glutamine is an unexpected phenomenon which calls for more extended study, as no similar exudation of asparagine has so far been recorded. The concentration of glutamine in the sap of the leaf cells is very much less than that of asparagine, *e.g.* in the sap of etiolated lupins, and since the solubility of glutamine in water, and therefore presumably in the cell sap, is greater than that of asparagine, the exudation cannot be ascribed to limiting solubility. It appears more probable that glutamine, even in relatively dilute solution, exercises some selective influence on the permeability of the rye-grass leaf cells, and is thereby able to diffuse outwards to the surface of the leaf, on which it is deposited in solid form by evaporation.

Now the rôle of asparagine in plant metabolism has been the subject of a large number of investigations. Schulze pointed out that the amount of aspartic acid yielded by plant proteins on acid hydrolysis was too small to permit the assumption—originally advanced by Pfeffer—that this amide was a primary product of protein metabolism. He showed that it was a secondary product of amino-acid oxidation, and it is now generally agreed that its formation, both in the case of protein breakdown and in the case of ammonia feeding through the root system, takes place from ammonia *via* the 4-carbon dicarboxylic acids, malic, succinic and fumaric.




The above scheme has, in fact, recently received striking confirmation from the experiments of Mothes [1933], who, using the vacuum infiltration feeding methods of Björkstén [1930], has been able to show that leaves of the runner bean, when fed with the ammonium salts of these three dicarboxylic acids, rapidly synthesise asparagine.

To the best of our knowledge no such searching enquiry has been made into the origin of glutamine. During the growth of etiolated seedlings or when leaves are kept in the dark, it is formed only to a limited extent, and it appears to us that there is evidence, which we shall present in greater detail in a later paper, for the assumption that it is a primary product of protein breakdown, for plant proteins yield on acid hydrolysis relatively large amounts of both glutamic acid and ammonia, while glutamine has been isolated from the enzymic digest of gliadin [Damodaran *et al.*, 1932]. Any suggestion therefore, that during etiolation the protein, fat or carbohydrate metabolism leads to the production of 5-carbon dicarboxylic acids, which, with ammonia, are the immediate precursors of



glutamine, is unnecessary. But the production of glutamine from ammonia absorbed through the root system shows that some such alternative and indirect synthesis must be possible in certain plants, and it is probable that this takes place *via* glutaconic, glutaric and α-hydroxyglutaric acids.

It is interesting to observe that both glutaric and α-hydroxyglutaric acids have been isolated from sugar beet, which also contains relatively large amounts of glutamine. Vacuum infiltration experiments to test this assumption, using blades of perennial rye-grass and the ammonium salts of these acids, are now in progress.

SUMMARY.

Under certain conditions perennial rye-grass when treated with ammonium sulphate produces a white exudation on the upper half of the blades. The exudation consists almost entirely of glutamine.

The bearing which this observation has on the metabolism of glutamine in the plant is briefly discussed.

We should like to record our thanks to Mr R. G. Westall for analytical assistance.

REFERENCES.

Björkstén (1930). *Biochem. Z.* **225**, 1.
Chibnall and Westall (1932). *Biochem. J.* **26**, 122.
Damodaran, Jaaback and Chibnall (1932). *Biochem. J.* **26**, 1704.
Greenhill and Page (1933). *J. Agric. Sci.* **23**, 329.
Mothes (1933). *Planta*, **19**, 117.
Stieger (1913). *Z. physiol. Chem.* **86**, 245.

# CXCI. THE VOLUMETRIC DETERMINATION OF INOSITOL.

By LESLIE YOUNG[1].

*From the Department of Physiology and Biochemistry, University College, London.*

(*Received June 30th, 1934.*)

THE lack of a simple method of estimation has greatly hindered the quantitative study of inositol, and most workers in this field have been obliged to adopt the laborious procedure of isolating and weighing the substance. A survey of the chemistry of inositol shows few reactions likely to be of value in attempting to evolve a method of estimation, and the difficulties encountered in endeavouring to apply quantitatively the colour tests and certain other reactions of inositol have been described by Needham [1923, 1]. One outstanding departure from the gravimetric method was due to Needham [1923, 1, 2], who determined the carbon content of the isolated material by a micro-combustion method.

Fleury and Marque [1929], as one of the analytical applications of potassium iodomercurate in alkaline solution, described a method of estimating polyhydric alcohols and related substances, including inositol. The oxidation of reducing sugars by potassium iodomercurate in hot alkaline solution was first applied analytically by Sacchse [1876], and Rupp [1905] made use of the reduction of iodomercurate in an alkaline medium to metallic mercury by the action of reducing compounds in his iodimetric method of estimating mercury. This procedure was in turn adapted by Bougault and Gros [1922] for the estimation of aldehydes. In the case of glucose the amount of mercury reduced indicated that the alcoholic groups were also being attacked. This led Fleury and Marque to study the action of alkaline solutions of potassium iodomercurate on certain hydroxylic compounds containing no aldehyde groups. They showed that the reaction was such that a number of these compounds, inositol among them, could be estimated by a modification of the technique used by Rupp [1905]. The course of the reaction was found to be dependent upon the concentration of potassium iodomercurate, the degree of alkalinity and the time of heating, for all of which they determined the optimum conditions.

The procedure adopted by Fleury and Marque [1929] involves heating the solution of the substance to be estimated at the temperature of the boiling water-bath with solutions of potassium iodomercurate and sodium hydroxide, with the addition of a suspension of barium sulphate. As the oxidation of the substance proceeds metallic mercury is deposited in the layer of barium sulphate at the bottom of the reaction flask. After a given period of heating the mixture is cooled and acidified, and standard iodine solution is added. Only a comparatively small excess of iodine is required to bring the mercury into solution on gently rotating the flask. This is due to the very finely divided form in which it has been maintained by the barium sulphate. Finally the excess of iodine is titrated with sodium thiosulphate solution.

[1] Bayliss-Starling Memorial Scholar.

With their method Fleury and Marque found that 10 mg. of anhydrous inositol are equivalent to 7·34 ml. of 0·1 *N* iodine solution (calculated from all values obtained for different quantities of inositol from 1 to 20 mg.). The results which have been obtained by this method compared with those recorded by Fleury and Marque [1929] are as follows:

| | Equivalent titrations—ml. of 0·1 *N* sodium thiosulphate (or iodine) solution | |
|---|---|---|
| mg. of inositol | Fleury and Marque | Present author |
| 1 | 0·67 | 0·67 |
| 5 | 3·67 | 3·70 |
| 10 | 7·39 | 7·41 |
| 15 | 11·03 | 11·04 |
| 20 | 14·33 | 14·49 |

In making quantitative studies of inositol in biological material [Young, 1934] it became necessary to estimate small amounts of inositol with much greater accuracy than is permitted by the original procedure. On account of this the Fleury-Marque method was modified in order to estimate 1–5 mg. of inositol more accurately than was hitherto possible. The details of this method, which embodies essentially the same technique as that of Fleury and Marque, are described below (I).

Owing to the fact that the iodomercurate method can also be used for estimating glucose it was felt desirable to make an investigation of the determination of inositol in the presence of this substance. Since the two compounds fractionate in a similar manner, it was not found possible to separate them by precipitation methods. By subjecting the mixture of the two substances to yeast fermentation it was found possible to recover the inositol after breakdown of the glucose, and the details of this work are given in a later section of this paper (II).

## I. The modified method of estimating inositol.

The quantities and strengths of reagents used in the modified method compared with those employed in the original are as follows:

| | Original method ml. | Modified method ml. |
|---|---|---|
| Inositol solution | 7·5 | 5·0 |
| Potassium iodomercurate solution | 7·5 | 3·0 |
| Sodium hydroxide solution | 10·0 | 4·0 |
| Barium sulphate suspension (10 %) | 10·0 | — |
| Barium sulphate suspension (20 %) | — | 2·0 |
| Sulphuric acid solution | 20·0 | 8·0 |
| Iodine solution (0·1 *N*) | 20·0 | 5·0 |

In place of the 0·1 *N* sodium thiosulphate solution used for titration of the excess of iodine by Fleury and Marque, 0·02 *N* solution is used in the modified method.

It will be seen that the same concentrations of iodomercurate and alkali have been retained. In order to determine the optimum period of heating a series of time experiments was performed with 2·5 mg. and 5·0 mg. of inositol. The results (Fig. 1) indicate that the reaction is almost complete at the end of 45 minutes' heating in a boiling water-bath. The rate of reaction after this period is so slow that small errors in the time of heating and subsequent rate of cooling of the reaction flask and its contents have no significant effect on the results of the estimation.

The equivalent titrations for 1–5 mg. of inositol obtained by three observers[1] using the modified method and working with independently prepared reagents are given below, each of the mean figures in the last column being obtained from at least twelve determinations.

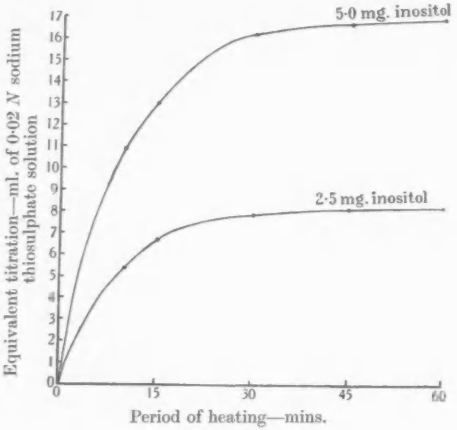



Fig. 1.

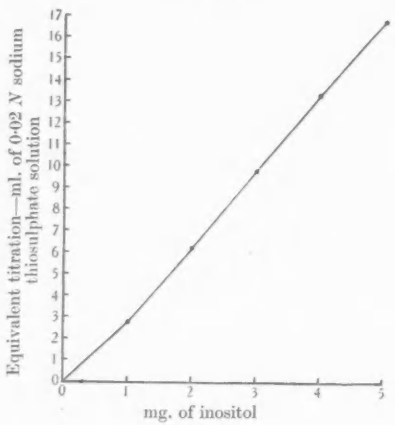



Fig. 2.

| mg. of inositol | Equivalent titrations—ml. of 0·2 *N* sodium thiosulphate solution | | | |
|---|---|---|---|---|
| | L. Y. | J. C. | F. G. K. | Mean |
| 1 | 2·75 | 2·76 | 2·85 | 2·79 |
| 2 | 6·35 | 6·28 | 6·25 | 6·29 |
| 3 | 9·90 | 9·90 | 9·80 | 9·87 |
| 4 | 13·38 | 13·47 | 13·25 | 13·37 |
| 5 | 16·72 | 16·82 | 16·73 | 16·76 |

[1] The author is indebted to Dr J. Chapman and Mr F. G. Kent for their assistance in this matter.

The relationship between the equivalent titration and weight of inositol estimated is illustrated in Fig. 2. It yields a graph which closely approximates to a straight line over the range 1–5 mg.

*Reagents.* The following reagents are used in the estimation:

Potassium iodomercurate solution. A solution of 288 g. of KI in water is added with shaking to 108 g. of mercuric chloride in water and made to 1 litre. The greenish yellow-coloured solution obtained is filtered unless perfectly clear.

Sodium hydroxide solution. 300 g. of pure sodium hydroxide are made to a litre with water. This solution is stored in an automatic Schellbach burette with the usual precautions.

Barium sulphate suspension. 20 g. of finely powdered barium sulphate (the form marketed as "Pure for X-ray examinations") are suspended in 80 ml. of water. This must be well shaken immediately before use.

Sulphuric acid solution. 200 ml. of concentrated acid are made to 1 litre with water.

0·1 *N* iodine solution.

0·02 *N* sodium thiosulphate solution. Prepared by diluting 0·1 *N* solution daily as required.

Starch solution.

Inositol solutions. Owing to the fact that hydrated inositol ($C_6H_{12}O_6$, $2H_2O$) is efflorescent, all standard inositol solutions are made from the anhydrous material. This is readily obtained from the crushed crystalline hydrate by heating it at 100–110° until constant in weight.

*Procedure.* Into a dry 100 ml. pyrex conical flask are pipetted 5 ml. of the solution to be estimated, containing not more than 5 mg. of inositol. Next, 3 ml. of the potassium iodomercurate reagent are introduced followed by 4 ml. of the 30 % NaOH solution. Lastly, 2 ml. of the well shaken barium sulphate suspension are added and the contents of the flask mixed by gentle rotation. The flask is firmly clamped in a level position in a boiling water-bath with the level of the water of the bath well above the level of the contents of the flask. As the reaction proceeds a red precipitate forms first and is soon replaced by a grey-black precipitate of mercury. After 45 minutes' heating the flask is removed and clamped in a bath of running water. After cooling for 5 minutes 8 ml. of the 20 % $H_2SO_4$ solution are added without moving the flask. After 5 minutes longer in the cooling bath the flask is removed and 5 ml. of 0·1 *N* iodine solution are measured into it. By carefully rotating the flask at intervals during a period of 10 minutes the mercury is readily brought into solution. Any undissolved mercury can be seen as a grey-black precipitate collected at the centre of the bottom of the flask on viewing the flask from underneath immediately after rotating it. At the end of the 10 minutes the excess of iodine is titrated with 0·02 *N* sodium thiosulphate solution, freshly prepared starch solution being added as the end-point is approached. The presence of the barium sulphate does not interfere with the detection of the end-point. The result obtained by direct titration of 5 ml. of 0·1 *N* iodine solution is used for the calculation of the equivalent titration, since it is found to be identical with the value obtained in a blank estimation.

*Further experimental details.* A series of experiments revealed that the slight errors of measurement resulting from the use of a 10 ml. micro-burette (0·05 ml. graduations) for measuring the potassium iodomercurate solution and a Schellbach burette (0·10 ml. graduations) for the NaOH solution did not have any detectable effect on the estimations. It is advisable to use a wide-tipped 2 ml. pipette for the addition of the barium sulphate suspension. The sulphuric acid solution is added from a burette and a standard pipette is used for the iodine solution. Care must be taken to avoid any undue disturbance of the contents of the reaction flask from the commencement of heating until the addition of the iodine solution. The reagents should be checked against standard inositol solutions periodically. Under ordinary conditions any deviations found are small and when necessary calculated corrections can be applied.

## II. The estimation of inositol in glucose-inositol mixtures.

Since glucose can be estimated by the iodomercurate method described above, the components of a glucose-inositol mixture must be separated before the inositol can be estimated. This separation is not readily brought about by chemical means and in consequence of this a study was made of the removal of glucose from such mixtures by fermentation methods.

The use of yeast fermentation by a number of workers for the determination of true sugar in blood has led to the recognition of certain precautions which must be observed in such quantitative investigations. The subtraction of the "residual reduction" value obtained after alcoholic fermentation of blood from the "apparent sugar" value was introduced by Otto [1885] for the determination of true sugar in blood. The causes of the conflicting results obtained by various authors have been reviewed by Hiller *et al.* [1925] and Somogyi [1927]. Baker and Hulton [1933] have recently studied the removal of sugars from dilute solutions by fermentation. Wholly satisfactory results were not obtained until Somogyi [1927] drew attention to the need for preliminary purification of the yeast suspensions used in the fermentation. Somogyi purified his yeast by suspending the fresh commercial product in water and repeatedly washing it by centrifuging until a supernatant liquid was obtained which yielded no reduction with the sugar reagent.

In the present work it was decided to remove the yeast by filtration on a sintered glass filter and to follow this by washing the yeast under standard conditions. This procedure has the following advantages: (i) it avoids the need for correcting for the cell volume of the yeast, (ii) it yields the whole of the solution, (iii) it is rapid.

*Procedure.* The fermentation technique which was the outcome of the above considerations was as follows: 10 ml. of the solution to be fermented contained in a 30 ml. pyrex beaker covered by a watch glass were incubated at 37°, and 10 ml. of a 20 % yeast suspension at room temperature (prepared as described below) were added and the mixture incubated at 28°. The mixture was stirred at intervals by a glass rod left in the beaker, and after the requisite period the yeast was filtered by suction on a sintered glass filter (Schott and Gen., Jena. –1G4). The beaker was successively rinsed with three approximately equal portions of water, making 10 ml. in all, and the rinsings were passed in turn through the filter. The whole filtration process took about 5 minutes. The filtrate was evaporated to dryness on the water-bath to remove alcohol (which reduces the iodomercurate reagent), redissolved in a little water, evaporated dry again and finally dissolved in water and estimated.

*Preparation of the yeast suspension.* Fresh baker's yeast was suspended in five times its weight of water and centrifuged, and portions of the supernatant liquid were estimated by the modified iodomercurate method after filtration through the sintered glass filter. By this means it was found that a progressive decrease in the amount of reducing substances occurred during the first four or five washings and that after this the reducing value of further washings tended to approach an approximately constant value equivalent to about 0·1 mg. inositol per g. of yeast being washed. When this state in the washing process was reached the yeast was suspended in four times its weight of water and was ready for use. When not in use it was stored in the refrigerator and was allowed to come to room temperature before being employed in fermentation experiments. The water was centrifuged off and replaced at intervals as recommended by Somogyi [1927].

*The rate of fermentation of glucose.* In order to determine the rate of breakdown of glucose under the conditions described above a series of experiments was done with 10, 25, 50 and 100 mg. of glucose and 5, 10, 20 and 30-minute periods of fermentation. For each period a blank experiment was performed. The blanks were finally made to 50 ml. and 5 ml. portions estimated. The small reduction values which they gave are shown in the table below:

| Time of incubation mins. | Equivalent titration— ml. of 0·02 *N* sodium thiosulphate |
|---|---|
| 5 | 0·11 |
| 10 | 0·16 |
| 20 | 0·20 |
| 30 | 0·22 |

The residual reduction values obtained after fermentation of the glucose, corrected for the above blanks were as follows:

| mg. of glucose taken | mg. of glucose found after fermentation period of | | | |
|---|---|---|---|---|
| | 5 mins. | 10 mins. | 20 mins. | 30 mins. |
| 10 | — | — | — | — |
| 25 | 1·7 | — | — | — |
| 50 | 16·0 | — | — | — |
| 100 | 42·1 | 21·2 | 1·2 | 1·6 |

The residual reductions are expressed in terms of glucose, the equivalent titrations for 1–5 mg. of glucose by the modified iodomercurate method having been found to be as follows:

| mg. of glucose | Equivalent titration—ml. of 0·02 *N* sodium thiosulphate |
|---|---|
| 1 | 3·07 |
| 2 | 6·31 |
| 3 | 9·84 |
| 4 | 13·32 |
| 5 | 16·69 |

It will be noted that these values are very close to those obtained for the same weights of inositol.

*The action of yeast on glucose-inositol mixtures.* A recovery of 24·9 mg. was obtained in an experiment in which 25 mg. of inositol were incubated with yeast for 20 minutes under the conditions described above, and this showed that no significant loss of inositol occurred during the process. Experiments were then done in which solutions containing inositol (25 mg.) and varying amounts of glucose (10, 25 and 50 mg.) were submitted to the fermentation procedure for periods long enough to permit the breakdown of all the glucose. The results were as follows:

| Time of fermentation mins. | mg. of glucose taken | mg. of inositol taken | Residual reduction (expressed as mg. inositol) |
|---|---|---|---|
| 5 | 10 | 25 | 25·1 |
| 10 | 25 | 25 | 25·1 |
| 20 | 50 | 25 | 25·2 |

With a mixture of 25 mg. inositol and 100 mg. glucose (30 minutes' fermentation) the results were less satisfactory, a reduction equivalent to 26·1 mg. of inositol being obtained after correcting for the residual reduction resulting from 30 minutes' fermentation of this weight of glucose. In the case of glucose-inositol mixtures in which the glucose does not preponderate so largely the results indicate that accurate determinations of the amounts of inositol present can readily be performed by the application of a yeast fermentation technique such as that described above.

## Summary.

1. A modification of the Fleury and Marque method of estimating polyhydric alcohols and related substances is described which permits 1–5 mg. of inositol to be estimated with greater accuracy than is possible by the original procedure.

2. The estimation of inositol in glucose-inositol mixtures is considered, and a technique for the rapid fermentation of the glucose by yeast is described which

gives accurate results when applied to the estimation of inositol in mixtures containing as much as twice its own weight of glucose.

The author is indebted to Prof. J. C. Drummond for his interest in this work and for his helpful suggestions.

REFERENCES.

Baker and Hulton (1933). *Biochem. J.* **27**, 1040.
Bougault and Gros (1922). *J. Pharm. Chim.* [7], **26**, 5.
Fleury and Marque (1929). *J. Pharm. Chim.* [8], **10**, 241.
Hiller, Linder and Van Slyke (1925). *J. Biol. Chem.* **64**, 625.
Needham (1923, 1). *Biochem. J.* **17**, 422.
—— (1923, 2). *Biochem. J.* **17**, 431.
Otto (1885). *Arch. ges. Physiol.* **35**, 467.
Rupp (1905). *Arch. Pharm.* **243**, 300.
Sacchse (1876). *Sitzungsber. naturf. Ges. Leipzig*, 17.
Somogyi (1927). *J. Biol. Chem.* **75**, 33.
Young (1934). *Biochem. J.* **28**, 1435.

# CXCII. THE DETERMINATION OF INOSITOL IN ANIMAL TISSUES[1].

By LESLIE YOUNG[2].

*From the Department of Physiology and Biochemistry, University College, London.*

(*Received June 30th, 1934.*)

Many attempts have been made to devise a satisfactory method of determining the inositol content of animal tissues. The nature of inositol and the absence of a simple method of estimating the substance have both contributed to the difficulties of the task. From necessity nearly all work has been directed towards isolating and weighing the inositol. The methods which were the outcome of early work were nearly all dependent upon the fact that inositol is precipitated by basic lead acetate. This principle was introduced by Marmé [1864] and more recently has constituted a stage in the methods of Rosenberger [1908], Starkenstein [1909], Needham [1923, 1] and Winter [1934]. The general procedure usually adopted involved the deproteinisation of the extract with a reagent such as normal lead acetate followed by precipitation of the inositol with basic lead acetate and ammonia. This lead precipitate was decomposed with hydrogen sulphide and the inositol thrown out of solution with alcohol. Needham [1923, 1] combined this process with an acetone extraction of the tissue and used a micro-combustion technique [1923, 2] for determining the carbon content of the isolated material. Winter [1934] destroyed the tissue with hot KOH solution, interposed a precipitation by baryta in alcoholic solution after the basic lead acetate precipitation, recrystallised the product obtained by decomposition of the baryta precipitate and weighed the pure inositol so obtained.

The development of the Fleury-Marque method of estimating inositol described in the preceding paper [Young, 1934] has made it possible to review the whole question of the determination of inositol in biological material. Primarily it must be recognised that any reagents capable of oxidising a compound as stable as inositol must inevitably be able to oxidise a wide range of substances. In this respect, however, potassium iodomercurate in an alkaline medium is much less catholic in its action than a reagent such as potassium permanganate. Thus Fleury and Marque [1929] found that although it could oxidise many organic hydroxylic compounds it showed either no action or only a feeble one in the case of compounds containing carboxyl groups as well as hydroxyl. In view of its lack of specificity it was felt that the iodomercurate method could only be applied with safety to the determination of inositol in tissues by combining it with an isolation process. The application of the iodomercurate method made it possible to check accurately the quantitative nature of all the processes involved

[1] A preliminary account of the work described in this and the preceding paper was made the subject of a paper given at the Biochemical Society meeting held on November 17th, 1933 [Young, 1933].

[2] Bayliss-Starling Memorial Scholar.

in the isolation of inositol. Furthermore, it permitted the estimation of inositol in the presence of inorganic compounds, which is important on account of the insolubility of inositol in organic solvents.

The tissues chosen for detailed investigation were brain and heart-muscle, partly because of their widely different natures and partly on account of reports of their relatively high inositol contents.

## The isolation of inositol.

*The extraction process.* Scherer [1850], who first isolated inositol, used water for extraction purposes, and this procedure was widely used by the earlier workers. By grinding brain tissues with normal lead acetate and water Müller [1857] made an extract from which he was able to isolate inositol. Rosenberger [1908] based his method on the stability of inositol to hot alkalis and destroyed the tissue with boiling potash solution. Momose [1916], acting on a suggestion of Rosenheim's, found that inositol can easily be extracted from fresh brain by means of acetone. Inositol is insoluble in acetone but is readily soluble in the aqueous acetone formed as the tissue is dehydrated. By combining the acetone extraction with the classical method of isolation Momose [1916] was able to obtain more inositol from brain than by applying the extraction procedures used by Scherer and Müller. Needham [1923, 1] used the acetone method and found that with butcher's beef-muscle no further amounts of inositol were obtained by a second extraction with acetone.

On the basis of the work of Momose [1916] and Needham [1923, 1] it was resolved to use the acetone method of extraction. It was found that when the tissue after acetone extraction was subjected to treatment with 70 % acetone it yielded an extract which contained inositol. The following experiments were therefore made with brain and heart-muscle. The minced tissue (50 g.) was first extracted with its own weight (1·25 vols.) of acetone for 24 hours. With this proportion of acetone and the water of the tissue an approximately 70 % concentration of acetone results. After filtration of the first extract (A) a second extraction with 1·25 vols. of aqueous acetone (70 % acetone) was made for 20 hours. This was filtered (B) and the tissue residue after being moistened with 70 % acetone was pressed on a small press, moistened with 70 % acetone, pressed again, and all the expressed liquid collected and filtered (C). The residue was then broken up and extracted again for 24 hours with 70 % acetone. This extract was filtered (D) and the tissue residue was finally extracted with water for 24 hours (E). The inositol content of each of these fractions was determined and yielded the following results:

| | mg. inositol found | |
|---|---|---|
| Extract | 50 g. brain (Ox) | 50 g. heart-muscle (Sheep) |
| A | 36·7 | 44·1 |
| B | 16·0 | 23·0 |
| C | 3·0 | 3·1 |
| D | 2·6 | Trace |
| E | Trace | Trace |

Thus, while extract A contained the greatest proportion of the inositol, considerable amounts were obtained in extract B. Extracts D and E showed only a trace of reduction on estimation, by far the greatest amount being obtained in the case of extract D from the brain. From these results it appeared that no serious inaccuracies would be introduced by confining the extraction to processes A, B and C.

The use of boiling 10 % KOH solution for disintegration of the tissue in the recent work of Winter [1934] on the estimation of inositol in heart muscle is noteworthy. Preliminary experiments by the author have shown that as an alternative to Winter's treatment the destruction of the tissue by KOH solution (70 g. KOH, 100 ml. $H_2O$) at the temperature of the boiling water-bath can be made the first stage of a method of estimation.

*The precipitation of contaminating substances present in the extract.* Normal lead acetate was the reagent most commonly used in the past for the removal of nitrogenous material and other contaminating substances present in tissue extracts prepared for the isolation of inositol. During recent years considerable attention has been directed towards the development of reagents preliminary to the determination of sugar. Certain other reagents which have come into use for the precipitation of nitrogenous substances in tissue extracts, *etc.* were studied with a view to finding the most suitable.

The Patein and Dufau [1902] mercuric nitrate reagent was used by a number of workers for the removal of interfering substances before sugar estimation, and Benedict and Osterberg [1918] showed that the mercuric nitrate-sodium bicarbonate treatment of urine yielded filtrates containing almost no measurable nitrogen. The disadvantage of this reagent lies in the large increase in the salt content of the filtrate which it produces, and on account of this it was ruled out for the present purpose. Smith [1932], in her study of the determination of sugar in bacteriological culture media, found that precipitation by phosphotungstic acid removed a larger proportion of non-sugar reducing substances than tungstic acid or any of the recognised mercury reagents. West *et al.* [1929], working on the determination of true sugar in blood, described a method of preliminary treatment which involved the use of mercuric sulphate in sulphuric acid followed by neutralisation with solid barium carbonate. The procedure was recommended by the authors for the preparation of filtrates from hydrolysed tissues. The reagent was subsequently modified slightly when applied to urine [West and Peterson, 1932]. On the basis of these considerations it was decided to compare the extent to which the alkaline iodomercurate reagent was reduced by filtrates prepared from identical tissue extracts by means of the West *et al.* mercuric sulphate method, phosphotungstic acid in sulphuric acid, normal lead acetate and mercuric chloride [Johnson, 1887].

Complete extractions of weighed samples of tissue (ox-brain and sheep's heart-muscle) were performed by the acetone method described above, the acetone was removed by distillation, the residual solution was extracted with ether and the resulting aqueous solution was made up to a known volume. Measured volumes of this solution were then submitted to complete precipitation by the methods under consideration and after removal of the excess of reagents the filtrates were brought to a slightly acid condition and estimated by the modified Fleury-Marque method. ($H_2S$ was used for precipitating excess of mercury and lead and excess $H_2S$ removed subsequently; baryta solution was added to neutrality in case of the excess phosphotungstic acid.) The following results were obtained:

| Method tested | Reducing power of filtrate (expressed as mg. inositol) equivalent to 100 g. of Brain (Ox) | Heart-muscle (Sheep) |
|---|---|---|
| Mercuric chloride | 344 | 649 |
| Normal lead acetate | 318 | 601 |
| Phosphotungstic acid in sulphuric acid | 291 | 553 |
| Acid mercuric sulphate | 257 | 437 |

Determinations of the inositol content of the extracts gave results of 111·4 mg per 100 g. of ox-brain and 140·4 mg. per 100 g. of sheep's heart-muscle.

The results showed the acid mercuric sulphate to be the most effective reagent for the process under consideration and it was therefore studied further. Nine experiments with aqueous solutions of inositol (10, 25, 50 and 100 mg.) subjected to this precipitation gave an average recovery in the filtrate of 98·5 % of the inositol taken (limits 97·6–100 %). Five experiments with inositol added to brain and heart-muscle extracts showed an average recovery of 98·8 % of added inositol (limits 94–104 %). On account of these results and the rapidity with which it could be performed the acid mercuric sulphate-barium carbonate precipitation was adopted as a stage in the isolation process.

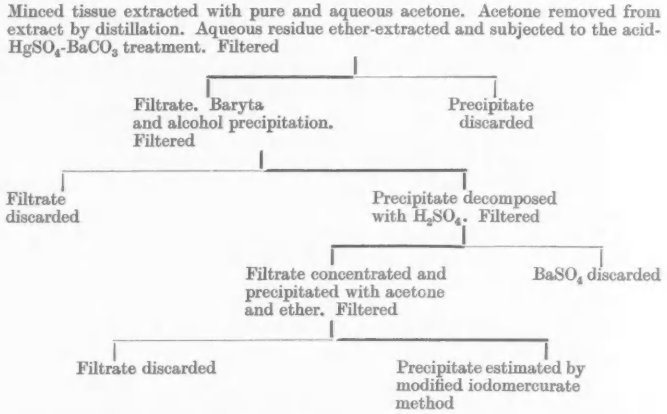
*The precipitation of inositol.* Basic lead acetate and ammonia were almost invariably used for this operation in the past. This procedure was not included in the isolation process under investigation since the use of ammonia is undesirable in view of the nature of the iodomercurate reagent. Thudichum [1901] described the precipitation of inositol by copper acetate but this was not found to be quantitative by Needham [1923, 1]. Buston and Pramanik [1931] reported the isolation of inositol from a baryta and alcohol precipitate obtained in the course of their work. The quantitative studies of this method by the author, described below, showed that it could be adapted for the present purpose.

The addition of alcoholic solutions of baryta to aqueous inositol solutions was found to give incomplete precipitation under the conditions tested, and saturation of the cold solutions with crystalline barium hydroxide followed by the addition of alcohol likewise gave unsatisfactory results. The solubility of barium hydroxide in water rapidly increases with rise of temperature, and it was felt that if a relatively large amount of this substance were to be dissolved in the hot aqueous inositol solution and then thrown out of solution by the addition of cold alcohol the chances of inositol being carried down by the precipitate might be enhanced. This was found to be so. Replacement of the cold alcohol by hot alcohol had little effect on the results. The inositol-baryta precipitate was found to be stable and showed only a slight tendency to dissociate on long standing (48 hours). Washing the precipitate with small amounts of cold absolute alcohol caused no detectable loss of inositol. An average precipitation of 96·3 % (limits 94·0–98·0 %) was found in twelve experiments with solutions (10 ml.) containing 10, 25 and 50 mg. of inositol. A slight excess of sulphuric acid was used to decompose the precipitates, and when this was done in hot solution followed by washing of the barium sulphate precipitate by hot water no loss of inositol by adsorption on the barium sulphate could be detected. In all the above experiments the solutions obtained after filtration of the barium sulphate were evaporated to dryness (after the addition of a slight excess of sodium carbonate solution) in order to remove traces of alcohol before estimation.

*The crystallisation of inositol.* The final stage of the isolation process is the throwing down of the inositol from solution in crystalline form. The earlier workers were accustomed to do this by addition of a large excess of alcohol with or without ether. Inositol is insoluble in acetone and since this liquid is employed in the extraction process it seemed that a purer product might be obtained by the use of acetone instead of alcohol for the crystallisation. An investigation was made of the precipitation of 10 and 25 mg. of inositol from 3 ml. of aqueous solution, and it was found possible to obtain 100 % precipitation of inositol in crystalline form by the addition of 30 ml. of acetone and 15 ml. of ether followed by refrigeration. The method was found to be equally effective when instead of using 3 ml. of neutral aqueous solution experiments were con-

ducted with 2 ml. of inositol solution together with 1 ml. of $2N$ $H_2SO_4$ or 1 ml. of glacial acetic acid.

*Summary of the process.* The method of determining inositol in tissues which was the outcome of the above work is as follows:


Minced tissue extracted with pure and aqueous acetone. Acetone removed from extract by distillation. Aqueous residue ether-extracted and subjected to the acid-$HgSO_4$-$BaCO_3$ treatment. Filtered
Filtrate. Baryta and alcohol precipitation. Filtered
Precipitate discarded
Filtrate discarded
Precipitate decomposed with $H_2SO_4$. Filtered
Filtrate concentrated and precipitated with acetone and ether. Filtered
$BaSO_4$ discarded
Filtrate discarded
Precipitate estimated by modified iodomercurate method

*The nature of the material isolated from tissues by the new method.*

Application of the method to brain and heart-muscle yielded good crystalline precipitates in the final stage, which on estimation behaved typically as inositol. The material showed no reduction with the Hagedorn-Jensen [1923] sugar reagent and either gave no Nessler precipitate or only a faint trace with the alkaline iodomercurate reagent. It gave positive Scherer [1850] and Fischler-Kürten [1932] tests for inositol. The crystals were not free from inorganic material and usually yielded incomplete melts, melting commencing at various temperatures from 210 to 219°. The ash content of the product estimated was lower when A.R. barium carbonate was used for neutralisation in the acid mercuric sulphate precipitation instead of the variety sold as "pure precip. by soda." The latter substance has a greater neutralising power for sulphuric acid than the former and this is probably due to its being contaminated by sodium carbonate. Two tissue estimations were performed using A.R. barium carbonate in the acid-mercuric sulphate precipitation and the products obtained by the complete precipitation of the solutions estimated were analysed (Schoeller).

(Found: Sample from brain (sheep) C, 39·74; H, 6·53; residue, 1·41; sample from heart-muscle (sheep): C, 39·92; H, 6·59; residue, 4·92; $C_6H_{12}O_6$ requires C, 40·00; H, 6·66 %.)

These results show that when the method is applied to brain and heart-muscle the product estimated is inositol. The contamination of the inositol by small amounts of inorganic compounds does not impair the accuracy of the iodomercurate estimation.

## EXPERIMENTAL.

The details about to be described are for the estimation of inositol in 20–40 g. of tissue.

*Extraction.* The finely minced tissue is weighed and extracted in a closed conical flask with its own weight (1·25 vols.) of acetone. After 24 hours' extrac-

tion with occasional shaking the liquid is poured off through a glass wool plug contained in a filter-funnel. The plug is washed with a few ml. of 70 % acetone and then transferred to the flask. After 20 hours' extraction the liquid is separated and the tissue residue pressed on a small press. It is then moistened with 70 % acetone and pressed again. The first and second acetone extracts and the expressed liquid are combined and filtered on a Büchner funnel. All washings are done with 70 % acetone.

*Distillation and ether extraction.* The acetone is removed from the extract by distillation on a gently heated water-bath. The aqueous extract which is left is evaporated and filtered (volume 100–150 ml.) and is extracted twice with equal volumes of ether, a few drops of alcohol being added when necessary to break the emulsions which may form. The combined ethereal extracts are extracted with half their volume of water. This washing is combined with the aqueous extract, heated gently to remove ether, concentrated by evaporation, filtered and made up to 100 ml. in a volumetric flask.

*The acid mercuric sulphate precipitation.* The aqueous extract is poured into a dry pyrex litre conical flask. The 100 ml. volumetric flask is then rinsed with two successive portions of 5 ml. of water and these in turn allowed to drain into the conical flask. From a burette are then added slowly with shaking 30 ml. of the acid-mercuric sulphate reagent [West and Peterson, 1932] prepared by dissolving 27 g. of mercuric sulphate in 100 ml. of 10 % (by weight) sulphuric acid at 5° and separating the solution from the slight precipitate which forms as room temperature is reached. Sufficient solid barium carbonate is then added to neutralise the mixture (60–70 g. of the material sold as "pure precip. by soda" or 160–170 g. of A.R. barium carbonate). The flask is rotated for a short time until most of the carbon dioxide has escaped. It is then stoppered and shaken vigorously, care being taken to release the pressure at intervals. When no more pressure develops the mixture is tested with blue litmus paper, and if necessary more barium carbonate added with shaking until the mixture does not turn blue litmus red. It is then filtered on a dry Büchner funnel into a dry flask under reduced pressure and a carefully measured amount (75–95 ml. of the filtrate, which is water-clear and colourless, is transferred to a 125 ml. Phillip's beaker. It is heated on the water-bath and made just acid with two or three small drops of concentrated sulphuric acid. A steady stream of $H_2S$ is then bubbled through the mixture for one minute and the precipitate of $BaSO_4$ and HgS filtered into a 250 ml. conical flask. The precipitate is well washed with hot water and $H_2S$ removed from the filtrate by adding a small fragment of porous pot and boiling on a sand-bath for a short time.

*The baryta and alcohol precipitation.* The solution in the conical flask is transferred to a 100 ml. beaker and concentrated on the water-bath to 10 ml. To the hot solution 3 g. of crushed crystalline barium hydroxide are added, the liquid is heated on the water-bath for 5 minutes with occasional stirring and 40 ml. of ethyl alcohol are then added slowly with vigorous stirring. At one point in the process the contents of the beaker appear to solidify. The precipitate is allowed to settle at room temperature for 2–3 hours and then filtered under reduced pressure on a sintered glass filter (Schott and Gen. Jena –3G3). The beaker is rinsed twice with 5 ml. of absolute alcohol, and the washings are passed in turn through the precipitate on the filter. When filtration is complete the precipitate is transferred by means of a small spatula to the original 100 ml. beaker. The small residue on the filter is dissolved in hot water and added to the main precipitate (volume 40–50 ml.). The beaker and its contents are heated on the water-bath, a slight excess of hot $2N$ $H_2SO_4$ is added and heating is con-

tinued for 45–60 minutes. The barium sulphate is filtered and washed with hot water until the volume of the filtrate (contained in a 250 ml. beaker) is about 125 ml.

*The acetone-ether precipitation.* The solution in the 250 ml. beaker is concentrated to 10–15 ml., transferred to a 30 ml. beaker, concentrated to about 3 ml., transferred to a 50 ml. pyrex conical flask and carefully concentrated on an electrically heated sand-bath to 3 ml. After cooling, 30 ml. of acetone and 15 ml. of ether are added, the sides of the flask are scratched with a glass rod to induce crystallisation, the flask is firmly stoppered and placed in the ice-chest for 24–36 hours. The precipitate which forms is filtered under reduced pressure on a sintered glass micro-filter (Schott and Gen. Jena. –12G3) and washed with acetone and ether. The conical flask and micro-filter are dried at 100–110° for a short time, and the material on the filter and also that adhering to the flask are dissolved in water, filtered if necessary and made up to a known volume.

*The estimation.* The volume of the solution for estimation should be such that the 5 ml. used for the modified iodomercurate estimation [Young, 1934] contain less than 5 mg. of inositol. The amount of inositol in the sample of tissue taken is obtained by multiplying the determined content of the final solution by the factor obtained by dividing 140 by the volume of filtrate taken after the acid-mercuric sulphate precipitation.

*Quantitative results.*

In order to trace the liberation of the inositol from contaminating substances by the various precipitations experiments were done in which iodomercurate estimations were performed after each stage. The results were as follows:

| | Reducing value (expressed as mg. inositol per 100 g. tissue) | |
|---|---|---|
| | Brain (ox) | Heart-muscle (sheep) |
| After acid-mercuric sulphate precipitation | 330 | 522 |
| After baryta and alcohol precipitation | 170 | 226 |
| After acetone and ether precipitation (inositol) | 149 | 154 |

It had been hoped that the isolation process might be shortened by the omission of the acetone and ether precipitation, but the above results showed that this was not possible.

As a test of the quantitative nature of the method an aqueous solution of 20 mg. inositol was mixed with acetone so as to give a 70 % concentration of acetone and this was submitted to the whole process. The estimation indicated 18·0 mg. as the amount of inositol originally taken. A series of experiments was then conducted with different tissues in order to find the loss of inositol which occurs during the process. Equal weights (A and B) of the sample of minced tissue were treated identically except that a known amount of inositol was added

| Exp. no. | Tissue | Weights taken g. (A and B) | mg. inositol added to B | Inositol found A | Inositol found B | % recovery inositol |
|---|---|---|---|---|---|---|
| 1 | Brain (ox) | 80 | 50 | 118·9 | 165·6 | 93·4 |
| 2 | Brain (sheep) | 40 | 25 | 70·3 | 93·7 | 93·6 |
| 3 | Brain (sheep) | 20 | 25 | 34·4 | 57·4 | 92·0 |
| 4 | Heart-muscle (sheep) | 50 | 50 | 76·9 | 121·0 | 88·2 |
| 5 | Heart-muscle (dog) | 20 | 25 | 31·1 | 52·7 | 86·4 |
| 6 | Skeletal muscle (ox) | 20 | 25 | <1·0 | 21·5 | — |
| 7 | Skeletal muscle (rabbit) | 40 | 25 | 10·9 | 32·3 | 85·6 |

to one portion (B) of the tissue before extraction. The average recovery of the added inositol in the experiments recorded below was 89·9 % (Exp. 6 is excluded from this calculation).

The determinations of the inositol content of several tissues were as follows:

| Tissue | mg. inositol per 100 g. tissue | | |
|---|---|---|---|
| Brain (ox) | (1) 149 | (2) 111 | |
| Brain (sheep) | (3) 176 | (4) 172 | |
| Heart-muscle (sheep) | (5) 154 | (6) 157 | (7) 170 |
| | | (8) 140 | (9) 158 |
| Heart-muscle (dog) | (10) 156 | (11) 174 | |
| Skeletal muscle (rabbit) | (12) 16 | (13) 27 | |
| Skeletal muscle (ox) | (14) less than 5 | | |

Exps. (3) and (4) were performed with different weights (20 and 40 g.) of the same specimen of brain and Exps. (5) and (6) with equal weights (50 g.) of the same specimen of heart-muscle. They serve to show the reproducibility of results which the method permits. The amounts of material obtained in the skeletal muscle, Exps. (12), (13) and (14), were insufficient for satisfactory qualitative study. The method was found to be unsuitable for application to liver.

In view of the work of Winter [1934] on the *post mortem* changes in the inositol content of dog's heart-muscle it should be pointed out that all the determinations recorded above were performed on tissues which had been removed from the animals at least several hours previously. Whether or not the inositol content of the tissues had reached a steady state before the determinations were started is not known. Winter reported values up to 185 mg. inositol per 100 g. dog heart-muscle and this maximum figure is close to the results of Exps. (10) and (11). In the case of brain very few quantitative data can be found in the literature, but it is of interest to note that Thudichum [1901] reported the inositol content of the grey matter of human brain to be 193 mg./100 g. and of the white matter to be 217·1 mg./100 g., results which are somewhat higher than those found for ox and sheep brains in the present work.

## Summary.

The modified Fleury-Marque method of estimating inositol has been used to study the behaviour of inositol with various precipitating reagents. From the results obtained a new method of determining the inositol content of tissues has been evolved and a detailed investigation of its application to brain and heart muscle has been made.

The author wishes to thank Prof. J. C. Drummond for his encouragement and advice. He is also indebted to Dr J. Chapman and Mr F. G. Kent for their kindness in checking some of the observations recorded herein.

## REFERENCES.

Benedict and Osterberg (1918). *J. Biol. Chem.* **34**, 195.
Buston and Pramanik (1931). *Biochem. J.* **25**, 1656.
Fischler and Kürten (1932). *Biochem. Z.* **254**, 138.
Fleury and Marque (1929). *J. Pharm. Chim.* [8], **10**, 241.
Hagedorn and Jensen (1923). *Biochem. Z.* **135**, 46.
Johnson (1887). *Proc. Roy. Soc. Lond.* **62**, 365.
Marmé (1864). *Liebig's Ann.* **129**, 222.

Momose (1916). *Biochem. J.* **10**, 120.
Müller (1857). *Liebig's Ann.* **103**, 140.
Needham (1923, 1). *Biochem. J.* **17**, 422.
—— (1923, 2). *Biochem. J.* **17**, 431.
Patein and Dufau (1902). *J. Pharm. Chim.* **15**, 221.
Rosenberger (1908). *Z. physiol. Chem.* **56**, 373.
Scherer (1850). *Liebig's Ann.* **73**, 322.
Smith (1932). *Biochem. J.* **26**, 1459.
Starkenstein (1909). *Z. exp. Path. Ther.* **5**, 378.
Thudichum (1901). Die chemische Konstitution des Gehirns des Menschen und der Tiere. (Tübingen.)
West and Peterson (1932). *Biochem. J.* **26**, 1720.
—— Scharles and Peterson (1929). *J. Biol. Chem.* **82**, 137.
Winter (1934). *Biochem. J.* **28**, 6.
Young (1933). *Chem. Ind.* **52**, 953.
—— (1934). *Biochem. J.* **28**, 1428.

# CXCIII. THE EFFECT OF VARIATIONS IN PROTEIN AND SALT INTAKE ON THE NITROGEN AND CHLORIDE CONTENT OF SWEAT.

By DAVID PATON CUTHBERTSON
and WILLIAM STEELE WATSON GUTHRIE.

*From the Biochemical Laboratory, Institute of Pathology, Royal Infirmary and University, Glasgow.*

(*Received June 27th, 1934.*)

Previous work on sweat and its composition has mainly been concerned with the quality and quantity of the various solutes secreted during short periods of profuse sweating induced by heat. Mosher [1933] has recently reviewed this aspect of metabolism.

Less numerous experiments have been made to determine the daily output of the sweat constituents at ordinary air temperatures, and, in particular, the effect of diet.

Most observers have determined the daily output of nitrogen and chloride by analysing extracts of the underclothing worn by their experimental subjects. With subjects at rest the average daily values for nitrogen were 0·071 g. [Benedict, 1905], 0·128 g. [Cramer, 1890], 0·286 g. [Graham and Poulton, 1912] and 0·33 g. [Schwenkenbecher and Spitta, 1907]. In tropical climates the nitrogen excretion is considerably increased [Eijkman, 1893]. When work is done the output of nitrogen may be as much as 0·22 g. per hour [Benedict, 1905].

Schwenkenbecher and Spitta [1907] recovered on an average 0·33 g. NaCl per day from the clothing of subjects at rest in bed. Cramer [1890] found a maximum of 2·27 g. per day secreted in the sweat of subjects actively employed.

The effect of variations in the amount of dietary protein on the composition of sweat was studied by Berry [1916], who induced sweating by cycling on an ergometer for periods of four hours. He found that the percentage composition of the sweat as regards nitrogen and chloride underwent no change on varying the protein content of the diet. The total amount of sweat produced was estimated from a calculation of his total water metabolism for the period of four hours. He found that the sweat-N corresponded to 2·0–5·6 % of the total N excreted in urine and sweat. Sweat-Cl corresponded to 13–30 % of total Cl excretion. This percentage increased both for N and Cl as the total protein metabolism decreased.

Bost and Borgstrom [1926] found that in two subjects doing office work and walking in the morning and evening, the average air temperature being 29·4° and the relative humidity 67 %, the excretions of nitrogen in the sweat during 24 hours were 0·900 g. and 0·613 g. The outputs per sq. m. body surface were respectively 0·522 g. and 0·353 g. per day. The nitrogen intakes were respectively 9·8 g. and 16·1 g. (61 and 101 g. protein). These writers concluded from other experiments lasting 2·5 to 3·75 hours that the protein content of the food eaten

in the twelve hours previous to the experiment did not seem to alter to any appreciable extent the amount of nitrogen excreted per sq. m. body surface per hour.

As for the effect of changes in the chloride content of the diet Kittsteiner [1911] asserted that systematic addition of salt to the diet increased the NaCl content of the sweat and *vice versa*, but that changes in diet of short duration had no effect.

The present investigation represents an attempt to determine the effect of variations in dietary protein and chloride on the composition of the sweat of resting subjects, care being taken that in each experiment nitrogen and, as far as possible, chloride equilibrium was first established. As has already been indicated, previous experiments have been concerned with the effect of variations in dietary protein of short duration and with the subjects not at rest. The writers thought that the extensive cutaneous excretion induced under these conditions might possibly mask such effects as could have been produced by diet alone. It was for this reason that the present experiments were conducted with the subjects lying in bed.

## Experimental methods.

The duration of each period of sweat collection was 48 hours in all the normal subjects and in two of the abnormal cases. In five of the eight abnormal subjects the period was 72 hours. In one case (exophthalmic goitre) sweating was so profuse that for the subject's well-being 24 hours were thought sufficient.

The experimental subjects were divided into a normal group and an abnormal group.

(i) *Normal group.* Subjects I–IV.

These were four healthy young males aged from 17 to 26 years. Though they had all been unemployed for several months before the experiments, they were in good condition due to participation in some form of sport.

Subjects I and II were placed first on a high protein + normal salt diet (A), then on a low protein + normal salt diet (B) and finally on a low protein + low salt diet (C). Subjects III and IV were placed on the same types of diet but the order was reversed.

*Diet A.* High protein (175–187 g. per day). Normal salt (11–12 g. per day).

The excess protein in these otherwise ordinary diets was made up by giving 500 g. meat and 400 g. egg-white per day. All food was cooked without salt. Saltless bread and fresh butter were used. 5–6 g. NaCl were added to bring the salt intake to a normal level.

*Diet B.* Low protein (67–77 g. per day). Normal salt (11–12 g. per day).

The diet consisted of saltless bread, fresh butter, milk, eggs, bran, potatoes, carrots, peas, sugar and tea. No meat was given. As before salt was dispensed with in cooking and 5–6 g. added each day.

*Diet C.* Low protein (67–77 g. per day). Low salt (5–6 g. per day). This diet was identical with *B* except that the extra salt was omitted.

Subjects II and III had exactly similar diets throughout the experiments. Slight modifications were made in those of Subjects I and IV, according to appetite. Throughout each period the same diet was adhered to from day to day. A constant fluid intake was observed.

Daily estimations of the urinary output of total N and NaCl were made. When there was less than 1 g. of difference between the urinary N outputs on two successive days, and the urinary excretion of NaCl was at a relatively constant level, the collection of sweat was commenced. In Exp. 1 B, however, considerable

variations were found in the urinary output of chloride even at the end of 13 days with approximately the same chloride intake.

The durations of the various diets prior to collection of sweat were as follows:

| Diet<br>Subject | *A*<br>days | *B*<br>days | *C*<br>days |
|---|---|---|---|
| I | 5 | 4 | 6 |
| II | 5 | 5 | 11 |
| III | 5 | 5 | 10 |
| IV | 4 | 7 | 8 |

(ii) *Abnormal group.* Subjects V–XII.

This group consists of:

(*a*) 2 males recovering from injuries.

V—Fracture of tibia (3–5 weeks after accident).

VI—Lacerated ankle with severed tendo Achillis (1–2 weeks after accident).

(*b*) 2 female cases of muscular dystrophy (VII and VIII).

(*c*) 2 cases of exophthalmic goitre, 1 female (IX); 1 male (X).

(*d*) 2 female cases of myxoedema (XI and XII).

*Diet.* In all these abnormal subjects sweat collection was effected while on a diet of low or moderate protein content (53–96 g. per day). In only four cases was it possible to arrange for a second collection when on high protein diet (117–240 g. per day). No account was taken of the intake of salt, the patients salting their food according to their taste. The intake of sodium chloride may therefore be taken as "normal." The subjects had the same diet for 4 to 7 days before sweat collections, except in Exps. V A, VI A and VII B where the periods were 21 days, 14 days and 3 months respectively.

*Method of collection of sweat.*

A roomy one-piece suit of calico was obtained with gloves and socks attached. All openings were fastened by tapes so arranged that the two sides overlapped. A loosely fitting jaconet suit constructed on somewhat similar lines was worn over this calico garment. While it is natural to suppose that such an arrangement must tend to prevent normal evaporation to a certain extent, yet some waterproof material was necessary to prevent loss of sweat on the bed linen.

A blank estimation was performed on control pieces of calico and jaconet of the same weight and quality as the suits. Each garment was boiled along with the control material in ordinary tap-water, to remove any loose matter and distribute evenly between the suit and control any traces of soluble substances still present. Both were washed out several times in the same changes of water and dried.

On the day the collection of sweat commenced, blood was withdrawn. The patient had no food in the morning prior to venepuncture. At 10 a.m. approximately, the subject had a bath, was sprayed down with distilled water and carefully dried. He donned the calico garment over which was worn the jaconet suit. The hands and feet were enclosed in both garments but the head and neck were left exposed. For two days (three in some of the abnormal cases) the subject remained in bed being covered only by a cotton sheet, a blanket and a counterpane.

During this time the urine was collected in 24-hourly lots. The greatest possible care was taken to avoid contamination of the garments with urine, the parts being washed after each passage. It was realised that a few ml. of urine of high nitrogen content voided on the clothing would lead to false results.

Wet and dry bulb hygrometer readings were taken every four hours, the instrument being attached to the head of the bed.

After 48 or 72 hours, as the case might be, the clothing was removed and the gain in weight noted. In most cases the clothing seemed quite dry except for the hands and feet. When the temperature was high or sweat secretion abnormally active, the calico garment was damp throughout and moisture was present on the inner surface of the jaconet suit.

The patient was washed down with 4 l. tap-water and dried with gauze towels which had been well boiled and rinsed. The clothing was placed in an enamelled bath containing the washings from the body and a further 8 l. tap-water added.

Control pieces of jaconet, calico and gauze towelling of the same weight and quality as those used for the subject were placed in a similar bath containing 12 l. tap-water. The contents of both baths were acidified with 5 ml. concentrated sulphuric acid.

As a control for evaporation 12 l. water were placed in a third similar bath.

After 24 hours' steeping, during which time the contents of the first two baths were stirred round from time to time, the clothing and control pieces of calico, jaconet and gauze were removed. Half the original volume, corrected for evaporation, was filtered in each case and concentrated to 1 l.

Analyses were then made on the extracts of (1) sweat and clothing, (2) clothing, and by subtraction values for the sweat were obtained.

*Analytical methods.*

*Blood-plasma.* Non-protein-N, Urea } Folin and Wu's colorimetric methods.
Chlorides (as NaCl): Van Slyke's method.

*Urine.* Total N: Kjeldahl.
(Urea + ammonia)-N: Aeration with urease incubation.
Chlorides (as NaCl): Volhard-Arnold method.

*Sweat.* The same methods were employed as for urine, 100 ml. of concentrated sweat washings being used in each case.

*Diet.* The values for the protein content of the diet were calculated from the tables of Sherman [1924].

The chloride content was determined on 3 g. of dried diet by the method of Sunderman and Williams [1933].

Talbert [1922] showed that sweat produced by work or heat is invariably acid and that when sweat is secreted under the same environmental conditions except temperature the work- and heat-sweats have the same $p_H$ (5·65 and 5·64 in his experiments). Sweat collected under rubber jackets is more acid, probably because there is not quite the same freedom for the escape of volatile organic acids. Mosher [1933] obtained $p_H$ values of 5·02 to 5·71 and McSwiney [1934] 5·1–7·35 with an average of 6·14 in 14 normal males.

It might be supposed that sweat collected in clothing over a period of two or three days would exhibit a tendency for its reaction to swing to the alkaline side due to decomposition. The wash-water from the body and clothing of our subjects gave with one exception (Exp. VI A) a $p_H$ of less than 6·8.

In the case where the sweat had become alkaline, the ward temperature was high (20·0°) and the subject's clothing was very moist. In this case there was no doubt that there was some escape of ammonia as the sweat (urea + ammonia)-N was only 20 % of the total N as against an average of 50 % in all the other

## Table I.

All values for intake, urine and sweat are in g. per day. Figures for urine and sweat are average excretions for 24 hours. The results for blood-plasma are in mg. per 100 ml.

| Condition | Normal | | | | | | | | | | | | Fracture | | Laceration | | Musc. dystrophy | | | Exoph. goitre | | | Myxoedema | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subject No. | I | | | II | | | III | | | IV | | | V | | VI | | VII | | VIII | IX | X | | XI | XII |
| Age | 17 | | | 26 | | | 22 | | | 26 | | | 17 | | 17 | | 31 | | 24 | 51 | 35 | | 46 | 33 |
| Sex | Male | | | Male | | | Male | | | Male | | | Male | | Male | | Female | | Male | Female | Male | | Female | Female |
| Experiment | A | B | C | A | B | C | C | B | A | C | B | A | A | B | A | B | B | A | B | B | B | A | B | B |
| Period of sweat collection (hrs.) | 48 | 48 | 48 | 48 | 48 | 48 | 48 | 48 | 48 | 48 | 48 | 48 | 72 | 72 | 72 | 72 | 72 | 72 | 48 | 72 | 24 | 24 | 72 | 48 |
| Surface area (sq. m.) | 1·52 | 1·52 | 1·52 | 1·78 | 1·82 | 1·82 | 1·64 | 1·65 | 1·63 | 1·79 | 1·75 | 1·76 | 1·44* | 1·44* | 1·33 | 1·34 | 1·65 | 1·65 | 1·25 | 1·24 | 1·68 | 1·68 | 1·51 | 1·53 |
| Av. room temp. (° C.) | 16·1 | 17·2 | 16·1 | 17·2 | 15·0 | 16·9 | 16·7 | 17·2 | 16·7 | 16·7 | 17·8 | 16·7 | 16·1 | 17·8 | 20·0 | 16·7 | 16·7 | 17·2 | 18·3 | 16·7 | 17·2 | 16·7 | 18·3 | 16·9 |
| *Nitrogen.* | | | | | | | | | | | | | | | | | | | | | | | | |
| Protein intake | 175 | 67 | 67 | 187 | 75 | 75 | 75 | 75 | 187 | 77 | 77 | 187 | 240 | 59 | 177 | 96 | 75‡ | 117 | 53‡ | 53 | 61 | 135 | 53 | 57 |
| Urine—total N | 24·32 | 10·92 | 11·48 | 24·36 | 9·80 | 10·78 | 10·78 | 10·22 | 23·52 | 10·36 | 9·94 | 25·20 | 26·43 | 11·53 | 22·54 | 12·72 | 11·06 | 14·14 | 8·40 | 6·30 | 17·78 | 24·50 | 8·09 | 6·84 |
| Sweat—total N | 0·458 | 0·307 | 0·227 | 0·465 | 0·215 | 0·412 | 0·231 | 0·249 | 0·328 | 0·336 | 0·370 | 0·431 | 0·388 | 0·305 | 0·440† | 0·262 | 0·221 | 0·221 | 0·224 | 0·270 | 1·019 | 1·126 | 0·059 | 0·062 |
| Sweat—total N/sq. m. | 0·301 | 0·202 | 0·146 | 0·261 | 0·118 | 0·226 | 0·140 | 0·151 | 0·201 | 0·188 | 0·211 | 0·245 | 0·270 | 0·212 | 0·331† | 0·196 | 0·133 | 0·133 | 0·179 | 0·218 | 0·606 | 0·607 | 0·039 | 0·041 |
| Plasma N-P-N | 39 | 25 | 32 | 49 | 32 | 31 | 24 | 26 | 29 | 26 | — | 40 | 46 | 28 | 29 | 30 | 33 | 28 | 33 | — | — | — | 25 | 22 |
| *Chloride (as NaCl).* | | | | | | | | | | | | | | | | | | | | | | | | |
| Intake | 11·42 | 11·21 | 5·21 | 12·17 | 12·27 | 5·77 | 5·77 | 11·77 | 12·17 | 5·63 | 11·29 | 11·17 | — | — | — | — | — | — | — | — | — | — | — | — |
| Urine | 9·77 | 14·51 | 4·64 | 9·95 | 10·53 | 5·38 | 5·73 | 11·23 | 12·75 | 3·91 | 9·20 | 10·76 | 10·53 | 8·39 | 15·91 | 8·39 | 7·80 | 8·89 | 6·49 | 7·37 | 5·85 | 8·77 | 3·69 | 3·05 |
| Sweat—total | 0·417 | 0·483 | 0·219 | 0·360 | 0·151 | 0·298 | 0·395 | 0·405 | 0·307 | 0·265 | 0·325 | 0·219 | 0·234 | 0·278 | 0·541 | 0·307 | 0·146 | 0·165 | 0·219 | 0·452 | 1·006 | 0·427 | 0·043 | 0·092 |
| Sweat—total/sq. m. | 0·274 | 0·318 | 0·144 | 0·202 | 0·083 | 0·164 | 0·241 | 0·246 | 0·188 | 0·148 | 0·186 | 0·125 | 0·155 | 0·184 | 0·407 | 0·229 | 0·088 | 0·099 | 0·175 | 0·364 | 0·600 | 0·254 | 0·028 | 0·060 |
| Plasma | 576 | 597 | 608 | 608 | 601 | 610 | 598 | 604 | 610 | 610 | — | 585 | 585 | 596 | 625 | 601 | 597 | 583 | — | — | — | — | 568 | 632 |

* Allowance made for surface area covered by plaster of Paris.

† Minimum values—probably loss of ammonia due to alkalinity of sweat.

‡ In addition to this allowance, 15 g. glycine (=2·8 g. N) given daily.

experiments. In a later experiment on the same subject, when the average temperature was 16·7° the sweat washings were acid. The (urea + ammonia)-N was then 66 % of the total N.

McSwiney [1934] found that in a sample of freshly voided sweat kept in a sterile bottle at 37°, the ammonia-N increased gradually from 3·8 mg. to 37·58 mg. per 100 ml. at 66 hours. He proved that this decomposition was mainly accomplished by the bacteria normally present on the skin. These organisms attack the urea and then the epithelial débris. For these reasons we lay no stress on results obtained for the absolute values of ammonia and urea in sweat collected over a period of 48 to 72 hours.

## Discussion.

It will be seen from Tables I and II that, irrespective of the nature of the diet, the N and NaCl content of the sweat per square metre body surface of

Table II. *Range of values (g. per day) irrespective of temperature and diet.*

| Condition | No. and Sex | Nitrogen | | Chloride (as NaCl) | | Basal metabolic rate |
|---|---|---|---|---|---|---|
| | | Total | Per sq. m. | Total | Per sq. m. | |
| (*a*) Normal | 4 M | 0·215–0·465 | 0·118–0·301 | 0·151–0·483 | 0·083–0·318 | — |
| (*b*) Healing injury | 2 M | 0·262–0·440 + * | 0·196–0·331 + * | 0·234–0·541 | 0·155–0·407 | — |
| (*a*) + (*b*) | 6 M | 0·215–0·465 | 0·118–0·331 + * | 0·151–0·541 | 0·083–0·407 | — |
| (*c*) Muscular dystrophy | 2 F | 0·221–0·224 | 0·133–0·179 | 0·146–0·219 | 0·088–0·175 | — |
| (*d*) Exophthalmic goitre | 1 F<br>1 M | 0·270–1·126 | 0·218–0·607 | 0·427–1·006 | 0·254–0·600 | +37 to +60% |
| (*e*) Myxoedema | 2 F | 0·059–0·062 | 0·039–0·041 | 0·043–0·092 | 0·028–0·060 | −33 to −47 % |

* Minimum values for Exp. VI A—some N probably lost owing to alkalinity of sweat.

*Note.* The surface areas of the subjects were calculated from their heights and weights by the formula of Du Bois and Du Bois [1916]. The values given for the output per sq. m. per day are lower than the actual values as the sweat from head and neck is not included. In Subject V (fracture of tibia) the area covered by plaster of Paris (0·069 sq. m.) was subtracted from the calculated total surface area (1·51 sq. m.) to give the value (1·44 sq. m.) in Table I.

Cases I–VI is 0·118–0·331 g. N and 0·083–0·407 g. NaCl per day at 15–20°. The upper figure 0·331 g. N (Exp. VI A) is, however, probably too low owing to loss of ammonia.

Increase in the weight of the clothing under the conditions of these experiments cannot be an accurate guide to the total weight of the sweat secreted. The greater part of the water of the sweat was found to evaporate and, in order to keep the experimental conditions as normal as possible, this evaporation was desirable.

The relative humidity obtaining in the ward during the experiments can be but a rough guide to the relative humidity of the air contained within the clothing, especially if sweating is profuse, owing to increased air temperature or pathological cause, *e.g.* exophthalmic goitre. We have therefore not included any figures for the relative humidity.

The following remarks apply only to the normal subjects I–IV where the conditions of experiment were comparable.

*The effect of increased temperature when the diet is constant.*

(i) *Protein.* Fig. 1 shows that with constant protein intake the excretion of total N per sq. m. per day rises with increase of air temperature.

(ii) *Chloride (as sodium chloride).* Fig. 2 indicates that the output of NaCl rises with increase of temperature if the salt intake is constant.

*The effect of increase in intake when the temperature is constant.*

(i) *Protein.* Exps. A and C, Subjects I–IV, were selected for comparison as the average temperatures during each pair of experiments were the same. This

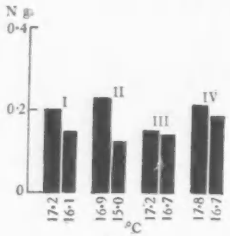



Fig. 1.

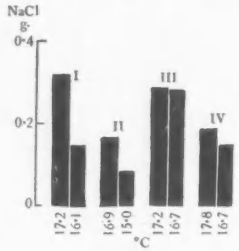



Fig. 2.

Fig. 1. Total sweat-N per sq. m. per day at different room temperatures but at same protein intake.

Fig. 2. Sweat-chloride (NaCl) per sq. m. per day at different room temperatures but at same chloride intake.

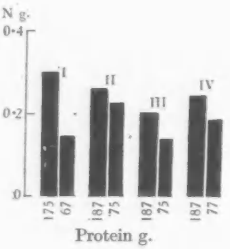



Fig. 3.

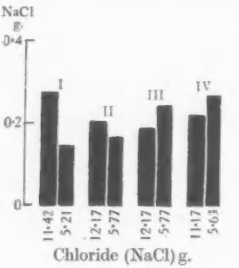



Fig. 4.

Fig. 3. Total sweat-N per sq. m. per day during high and low protein diets but at same average room temperature.

Fig. 4. Sweat-chloride (NaCl) per sq. m. per day during normal and low chloride diets, but at same average room temperature.

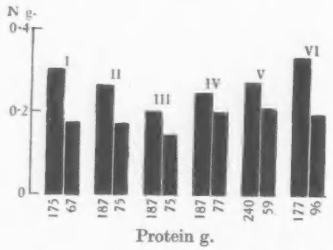



Fig. 5. Total sweat-N per sq. m. per day during high and during low protein diets (in subjects I–IV average of two low protein diets) and irrespective of room temperature.

similarity in the temperatures was quite fortuitous. No attempt was made to keep the ward temperature at a definite level. It is seen that a rise in protein intake increases the output of N through the skin (Fig. 3).

(ii) *Chloride.* Increase of salt intake, at least within the limits obtaining in these experiments, has no constant effect on the excretion of chloride in the sweat. In the four normal cases where the temperatures were the same for both experiments in each subject, comparison of the sweat-NaCl on diets C (low salt) and A (normal salt) reveals that in two cases increase in the chloride intake was followed by a rise in sweat-NaCl and in the other two cases by a fall (Fig. 4). Kittsteiner [1911] stated that increase of salt intake raised the chloride content of the sweat but not in experiments of short duration. It is possible that a positive result might have been obtained in the present experiments if they had been prolonged.

*The effect of variations in diet, the temperature being inconstant.*

(i) *Protein.* It will be observed from Table I and Fig. 5 that within the limits of these experiments increment in dietary protein has a greater effect than changes in air temperature, though, as has already been pointed out, these have a definite effect.

(ii) *Chloride* (*as sodium chloride*). In contrast to the preponderating effect of increases in dietary protein, increments in chloride intake do not possess this effect. As already indicated, temperature was the important factor in determining the output of chloride in these experiments.

The total values for (urea+ammonia)-N in Subjects I–VI while on high protein diets ranged from 0·232 to 0·260 g. per day, if we exclude Exp. VI A where the sweat was found to be alkaline. The (urea+ammonia)-N as a percentage of the total N gave an average of 60. On low protein diets in Subjects I–VI the limits were 0·106 and 0·209 g. per day and the average proportion of the total N as urea+ammonia was 51 %.

The fraction of total N in the urine present as urea+ammonia in these six subjects while on high protein diets averaged 83 %. The corresponding value for low protein was 84 %. Over this series of experiments the proportion of the total urinary N as urea+ammonia did not seem to have been affected by reducing the protein content of the diet. It is to be noted, however, that very low protein diets were not used, the smallest allowances in Subjects I–VI being 59 g. per day.

In the blood-plasma on high protein diets Subjects I–VI showed a variation in the non-protein-N from 29 to 49 mg. per 100 ml. with an average of 39 mg. per 100 ml. On low protein diets the range was from 24 to 32 mg. per 100 ml., the average being 28 mg. per 100 ml.

In Subjects II and IV the non-protein-N was definitely higher when on high protein diet. In Subjects I and III, however, the two values when on low protein diet showed variations practically as great as the differences between the higher figures for low protein and the values for high protein.

The urea-N of the blood as a percentage of the non-protein-N averaged 53 on high protein diet and 54 on low protein diet.

The daily output of sodium chloride in the urine was naturally higher when the salt intake was increased. The plasma-NaCl, as was to be expected, remained at a fixed level for the individual, being unaffected by diet. No conclusions can be drawn from a study of the non-protein-N and chloride contents of the plasma at one particular moment as to the presence of a correlation between these values and the excretion in the sweat of nitrogen and chloride over a period of 48 hours.

It was not found possible to investigate normal females. The values for the female cases of muscular dystrophy (both thin persons) fall within the normal

male limits, if these can be used for comparison. In comparing Exps. III C (normal male) and VII B (dystrophic female) where the temperature and protein intake were exactly the same, we find that the output of N per sq. m. per day was 0·140 g. in III C and 0·133 g. in VII B. The corresponding values for NaCl at the same temperature were, however, 0·241 g. and 0·088 g. despite the fact that in the former the salt intake was lower. In Exp. VII B the patient was receiving as treatment 15 g. glycine per day (2·8 g. N). This lessens considerably the difference in total N intake between Exps. B and A and may account in part for the similarity in sweat-N excretion.

There is a considerable divergence in the results for the two cases of exophthalmic goitre. The basal metabolic rates before the experiments commenced were +37 % for Subject IX and +60 % for Subject X. The basal metabolic rate in Subject IX after one month's subsequent treatment with iodine was still +44 %. Subject IX, who was a comparatively mild case of exophthalmic goitre, had excretions of N and NaCl by the skin comparable with the normal male cases though considerably above the upper limit for the female subjects with muscular dystrophy.

Subject X was a very severe case of exophthalmic goitre. In the two experiments the values for total N were 0·606 and 0·607 g. per sq. m. per day. On diets of normal salt content, the values for the sweat-Cl (as NaCl) were 0·600 g. and 0·254 g. per sq. m. per day. Owing to the extreme nervousness of the subject, the volume of sweat was much greater in the first experiment (B)—360 g. increase in weight of clothing as against 180 g. in Exp. A. This difference is reflected in the results for sweat-chloride. On account of this variable psychic factor, no conclusions can be drawn with regard to the effect of diet on the sweat output of nitrogen in this case.

Magnus-Levy [1907] stated "In myxoedema even when the body is exposed to great heat, no sweat appears; this feature is very characteristic. Of course a certain degree of invisible sweating may occur. It is interesting to notice that the appearance of sweat on the surface of the body is nearly always the first sign of improvement." We have shown that in cases with a reduction of basal metabolic rate of 33 % (XI) and 47 % (XII) there was a slight secretion of sweat while resting in bed even before any treatment was given. The output of N per sq. m. per day was practically the same in both cases, 0·039 g. (XI) and 0·041 g. (XII). In the sweat of Subject XII the value for NaCl was twice that in XI but was still well below the lowest normal result.

## Summary.

1. When the dry bulb temperature was constant, high protein intake (175–187 g. daily) was accompanied by an increased daily output of nitrogen in the sweat of normal subjects as compared with their output on low protein intake (67–77 g. daily).

2. If the intake of protein was the same, increase in air temperature of the order described apparently led to a higher daily excretion of nitrogen in the sweat.

3. The effect of alterations in protein intake was in all cases in excess of that due to temperature. The variations in protein intake were, however, of a much greater order than the changes in air temperature.

4. At the same temperatures, increase in salt intake of the order described had no constant effect on the output of chloride in the sweat, at least where the subjects had the same intake of chloride for 6–13 days beforehand.

5. The output of chloride in the sweat rose with increase of air temperature if the intake of chloride was constant.

6. In two cases of myxoedema there was a greatly reduced output both of nitrogen and chloride in the sweat.

7. In two cases of exophthalmic goitre the output of total nitrogen and chloride appeared to vary with the severity of the disease as shown by the basal metabolic rate.

These investigations were commenced during the tenure by one of us (W. S. W. G.) of a McCunn Research Scholarship and continued with the aid of grants from the Medical Research Council (D. P. C. and W. S. W. G.). To the McCunn Trustees and to the Medical Research Council we tender our thanks. We are indebted to Prof. J. Shaw Dunn for his interest in this investigation and to the Clinical Staff of the Royal Infirmary for providing us with suitable cases. Our thanks are also due to Sister Cumming for her co-operation in supervising the dietary arrangements.

## REFERENCES.

Benedict (1905). *J. Biol. Chem.* **1**, 263.
Berry (1916). *Biochem. Z.* **72**, 284.
Bost and Borgstrom (1926). *Amer. J. Physiol.* **79**, 242.
Cramer (1890). *Arch. Hyg.* **10**, 231.
Du Bois and Du Bois (1916). *Arch. Int. Med.* **17**, 863.
Eijkman (1893). *Virchow's Arch.* **131**, 167.
Graham and Poulton (1912). *Quart. J. Med.* **6**, 82.
Kittsteiner (1911). *Arch. Hyg.* **73**, 275.
McSwiney (1934). *Proc. Roy. Soc. Med.* **27**, Sec. Phys. Med. 23.
Magnus-Levy (1907). Von Noorden's Metabolism and general medicine. Eng. ed. **3**, p. 988. (Heinemann, London.)
Mosher (1933). *J. Biol. Chem.* **99**, 781.
Schwenkenbecher and Spitta (1907). *Arch. exp. Path. Pharm.* **56**, 284.
Sherman (1924). Chemistry of food and nutrition. 2nd ed, 410. (Macmillan and Co., London.)
Sunderman and Williams (1933). *J. Biol. Chem.* **102**, 279.
Talbert (1922). *Amer. J. Physiol.* **61**, 493.

# CXCIV. THE DEHYDROGENASES OF *BACTERIUM COLI*.

## II. THE RATE OF REDUCTION OF METHYLENE BLUE.

By JOHN YUDKIN[1].

*From the Department of Colloid Science and the Biochemical Laboratory, Cambridge.*

(*Received June 30th, 1934.*)

In a previous paper of this series [Yudkin, 1933] it was shown that the reduction time of a quantity of methylene blue by a washed suspension of *Bact. coli* in the presence of a substrate depended on the interaction of several factors. These were:

1. The temperature lag effect, that is, the slower rate of reduction of the dye at the beginning of the experiment while the reactants are acquiring the temperature of the bath.
2. The presence of residual oxygen in the Thunberg tube.
3. The gradual poisoning of the enzyme by methylene blue.
4. The affinity of the enzyme for methylene blue.
5. The presence of hydrogen donators in the bacterial suspension.
6. The products of the action of the bacteria on the substrate which can act as further hydrogen donators.

The first four of these will tend to increase the reduction time, the last two to decrease it.

In this paper, the study of the rate of reduction of methylene blue was undertaken with a view to demonstrating more clearly the part played by each of these factors in determining the reduction time.

The rate of reduction of methylene blue is always held to be linear for the greater part of the reaction. In the case of the succinic dehydrogenase of *Bact. coli*, it was shown colorimetrically that the reduction is linear until about 90 % of the dye is reduced, after which the rate falls off [Quastel and Whetham, 1924]. This falling off in the rate when the dye concentration has become small is explained by the affinity of the dye for the enzyme: that is, the concentration of the methylene blue has reached a value below that at which it is able to saturate the enzyme.

The rate of reduction of methylene blue has also been studied electrometrically with yeast suspensions [Cannan *et al.*, 1926]. Here it is stated that the reduction is linear throughout.

### Experimental.

The preparation of the bacterial suspension and the reduction of methylene blue in Thunberg tubes were carried out as described in the first paper of this series. The same three enzymes were investigated, namely formic, succinic and glucose dehydrogenases. The rate at which the methylene blue was reduced

[1] Benn W. Levy Student.

was followed colorimetrically, the gradually decreasing depth of colour in the experimental tubes being compared with a series of standard tubes containing varying quantities of methylene blue, 1 ml. of the bacterial suspension being used in the experiment and water to bring the volume to 5 ml. [Quastel and Whetham, 1924].

All experiments were done at least in duplicate. With times of 20 minutes or more, duplicate values for any given degree of reduction usually agreed within 5 %, always within 10 %. The error was larger for smaller reduction times. However, on drawing a smooth curve through the points obtained from any pair of duplicates, these errors largely cancelled out, so that two pairs of duplicates done with exactly the same bacteria *etc.* gave curves which could be very well superimposed.

## Results.

### 1. *Rate of reduction of methylene blue by formic, succinic and glucose dehydrogenases.*

A typical series of results is shown in Fig. 1.

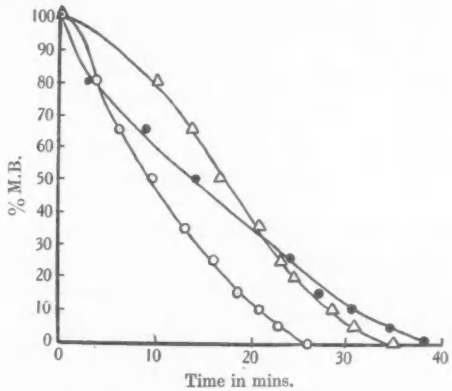



Fig. 1. Rate of reduction of methylene blue.
△ Formic dehydrogenase. • Succinic dehydrogenase. o Glucose dehydrogenase.

With succinic dehydrogenase is seen very well the effect of Factor 5 mentioned above—the acceleration due to hydrogen donators in the suspension. These are rapidly used up, giving a decreased rate of reduction. The linear reduction for the first 80 % and then a falling off obtained by Quastel and Whetham [1924] for the same enzyme might be due to the fact that their reduction time was much less—about 9 minutes. There would therefore be a greater error due to temperature lag and residual oxygen (Factors 1 and 2) which would act in the opposite direction to Factor 5, and would also be manifest in the earlier part of the experiment.

In the case of the other two enzymes, there is seen an initial lag in the rate of reduction, particularly with formic dehydrogenase. This is due to Factors 1 and 2. The lower magnitude of this effect in the case of glucose dehydrogenase, and its complete absence in the case of the succinic enzyme, may be explained by the fact that the suspensions of bacteria used for these two enzymes were much stronger. For the glucose enzyme it was about 10 times, and for the succinic

enzyme about 50 times more concentrated than for the formic enzyme. There were therefore more hydrogen donators in these suspensions which partially or completely masked the lag effects of temperature and oxygen.

The decreasing rate of reduction towards the end, due to the affinity of the enzymes for the dyestuff, is well shown for each of the three dehydrogenases.

2. *Effect of dilution on the rate of reduction.*

The rate of reduction with succinic dehydrogenase was followed with two suspensions of bacteria, one being one-third as concentrated as the other (Fig. 2).

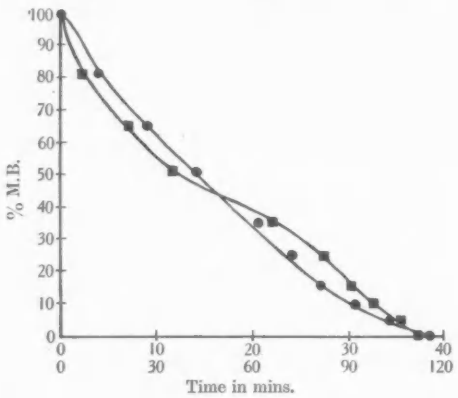



Fig. 2. Rate of reduction of methylene blue: effect of dilution. Succinic dehydrogenase. • Undiluted. ■ Diluted 1 in 3.

The scale of the abscissa for the curve with the diluted suspension is three times that of the more concentrated, so that the effects of dilution are shown more clearly.

The initial relatively more rapid reduction with the more dilute suspension may be accounted for by the relatively smaller lag effect here, where the actual time for reduction is greater. The rate with the diluted suspension then begins to fall off due to the poisoning with methylene blue (Factor 3). Towards the end of the experiment, however, the rate increases again before the final falling off. This seems to be due to an activation of the enzyme during this period at 40°. An indication of this phenomenon was obtained in the previous work, in which a slightly more rapid reduction occurred with succinic acid after the bacteria had been warmed for one hour at 40°, compared with bacteria kept at 0°. This is not due to a liberation of donators from the bacteria themselves since there is no corresponding decrease in reduction time in the controls without succinate after the bacteria had been kept at 40° for one hour.

The figure shows that the effect of dilution on the reduction time depends on the end-point chosen. If 80 % reduction is taken, the reduction time of a suspension diluted three times is rather more than three times that of the undiluted (85 minutes and 25 minutes). If complete reduction is taken, then the diluted suspension gives a reduction time rather less than three times that of the undiluted (112 minutes and 38 minutes).

The curves for glucose (Fig. 3) show not only the factors mentioned above but also the effect due to the coenzyme [Yudkin, 1933]. Although the bacteria in the diluted suspension were two-thirds as concentrated as in the undiluted, the scale of the abscissa for the results with the former had to be three times that of the latter in order to bring them together as shown.

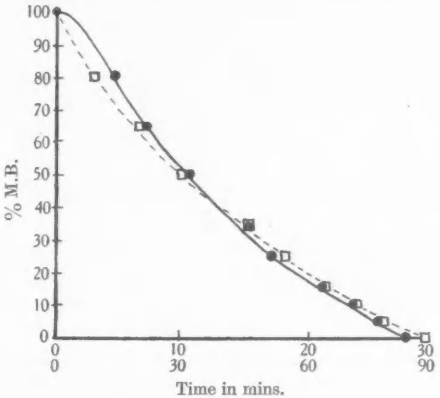



Fig. 3. Rate of reduction of methylene blue: effect of dilution. Glucose dehydrogenase.
• Undiluted. □ Diluted 1 in 3.

### 3. *Reduction of varying quantities of methylene blue.*

(*a*) *Times for complete reduction.* In order to avoid the prolonged action of methylene blue on the enzymes in experiments where the latter were unavoidably dilute, attempts were made to use smaller quantities of the dyestuff. The results obtained on taking the times for complete reduction of different quantities of methylene blue show very striking differences for the different enzymes. Succinic dehydrogenase shows a decreasing reduction time for decreasing amounts of methylene blue, whilst with formic acid, when the concentration of dye is below a certain value, the times taken to reduce very different quantities of the dye are approximately the same (Fig. 4). The results for glucose dehydrogenase are similar to those for formic dehydrogenase (Fig. 5).

These somewhat extraordinary results were found to be readily explicable by a consideration of the factors detailed above, which are involved in determining the time of reduction. These factors are well brought out by a study of the rates of reduction of these varying quantities of methylene blue.

With succinic dehydrogenase (Fig. 6), the rapid reduction of the small quantities of methylene blue is seen to be due in the main to the reduction of the dye by the other donators present (Factor 5). With higher dye concentration is seen the acceleration in the latter half of the reduction which we have suggested is due to an activation of the enzyme.

With the other two enzymes (Figs. 7 and 8) it is clear that the relatively long reduction time for the smaller quantities of dye can be explained by Factors 1–3. The first two of these factors—the lag due to residual air and temperature—will almost certainly have disappeared after the first 10 minutes. Yet it will be seen that the slow final rates of reduction with smaller quantities

of dye are much slower than the final rates of corresponding quantities of dye in those experiments where the initial concentration of dye was higher. Thus, when 1 ml. of methylene blue was used, the part of the curve showing the rate of reduction of the last 0·2 ml. of methylene blue is much steeper than the

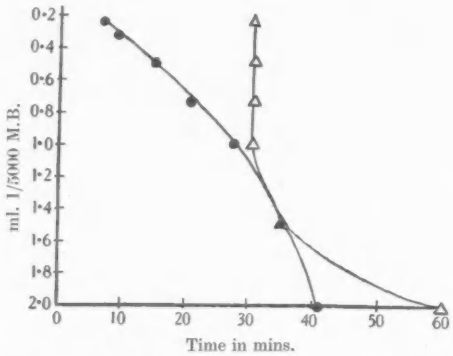



Fig. 4. Time for complete reduction of varying quantities of methylene blue.
△ Formic dehydrogenase. • Succinic dehydrogenase.

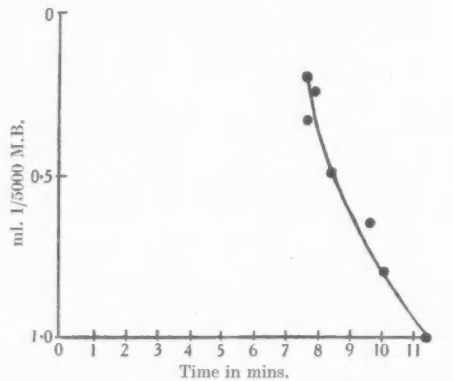



Fig. 5. Time for complete reduction of varying quantities of methylene blue. Glucose dehydrogenase.

corresponding part of the curve for the rate of reduction of 0·25 ml. These results may be explained by an accumulation of secondary products of the reaction between bacteria and substrate (Factor 6). This explanation was tested by experiments in which, after a certain quantity of dye had been reduced, a second quantity was added.

(*b*) *The effect of addition of a second quantity of methylene blue.* For these experiments, Thunberg tubes were used in which the second quantity of dye was contained in a side-arm. This was mixed with the other reactants immediately after the reduction of the first quantity of dye.

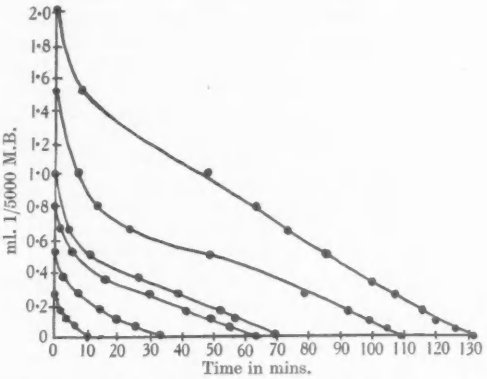



Fig. 6. Rate of reduction of varying amounts of methylene blue. Succinic dehydrogenase.

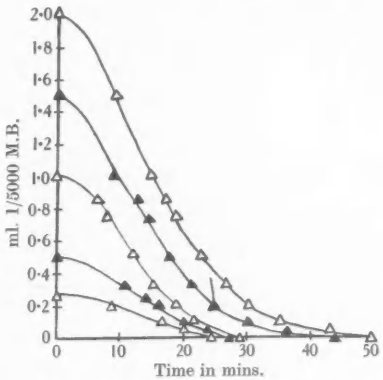



Fig. 7. Rate of reduction of varying amounts of methylene blue. Formic dehydrogenase.

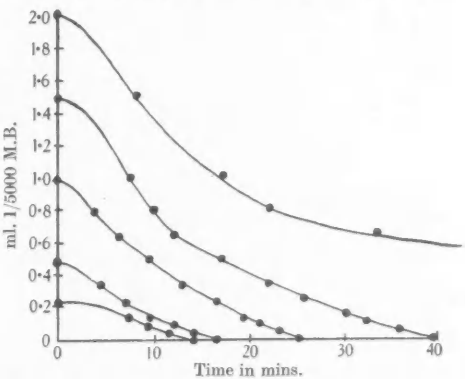



Fig. 8. Rate of reduction of varying amounts of methylene blue. Glucose dehydrogenase.

Formic and glucose dehydrogenases reduce the second quantity of methylene blue much more rapidly than the first, whilst succinic dehydrogenase does the reverse (Table I).

Table I.

| | Reduction time of 0·5 ml. M.B. in mins. | | Reduction time of 0·25 ml. M.B. in mins. | |
|---|---|---|---|---|
| Dehydrogenase | 1st 0·5 ml. | 2nd 0·5 ml. | 1st 0·25 ml. | 2nd 0·25 ml. |
| Formic | 26 | 13 | 25·5 | 9·5 |
| Glucose | 16·5 | 12 | 15·5 | 8 |
| Succinic | 43 | 73 | 20 | 30 |

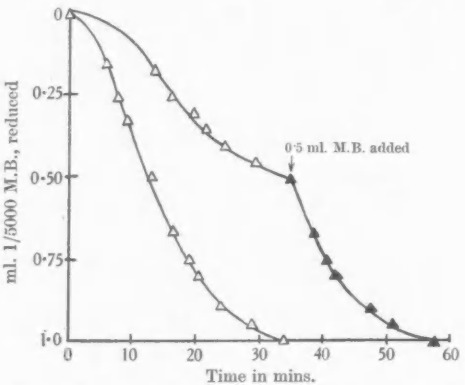



Fig. 9. Rate of reduction of methylene blue. Formic dehydrogenase.

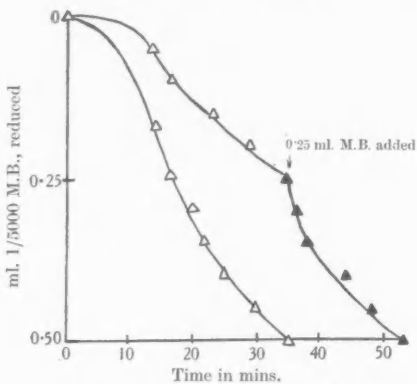



Fig. 10. Rate of reduction of methylene blue. Formic dehydrogenase.

The results with the first two enzymes might be due in part to the lag effects which, of course, manifest themselves only during the first half of each experiment. However, on following the rate of reduction it is seen that there is a real increased rate of reduction; the reduction now proceeds as if the whole amount of the dye had been present originally (Figs. 9 and 10). The curves after the

addition of the second amounts of dye can be well superimposed on the lower part of the curves showing the reduction of the double quantity of methylene blue. Exactly similar results were obtained with glucose dehydrogenase.

A more rapid reduction of a second quantity of methylene blue has also been shown for yeast [Ambrus *et al.*, 1931]. Here, however, it was shown to be due not to an increase in the quantity of hydrogen donators but to an increased penetration of the dye into the organisms.

The fact that succinic dehydrogenase gives a greater reduction time for the second quantity of methylene blue is to be expected from Fig. 6.

4. *Effect of addition of leucomethylene blue.*

It has been shown that an accelerated reduction occurs with the enzymes which is due to one or more of the products of reaction. It has hitherto been assumed that these are the products of the action of the bacteria on the substrate. However, the possibility existed that the other product of the reaction, namely leucomethylene blue, might produce this effect.

This hypothesis was tested in the following way (Fig. 11). Except for the rubber stoppers in the three boiling-tubes, the apparatus consisted entirely of glass. Methylene blue was reduced by a palladium catalyst, prepared by the precipitation of palladium on to barium sulphate by reduction with formaldehyde. Some dye, together with about 20 mg. of catalyst, was placed in each of tubes 1 and 2, and hydrogen passed until reduction was effected. This took about 10 minutes. Nitrogen, freed from oxygen by passing over palladium-copper heated to 380°, was passed for $\frac{1}{2}$–$\frac{3}{4}$ hour to remove the excess of hydrogen. Tube 1, with leucomethylene blue, served to indicate whether the nitrogen was in reality oxygen-free. Actually, no coloration took place after running the purified nitrogen for several hours. Leucomethylene blue was now drawn up into the burette, which had previously been freed of air by passing nitrogen.

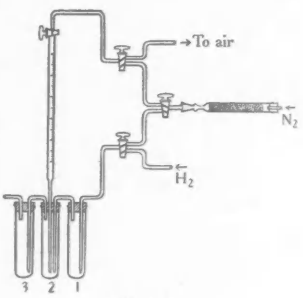


Fig. 11.

Two tubes, each containing equal quantities of buffer solution ($p_H$ 7·0) and substrate ($M/10$) were cooled to 0°, and then equal volumes of cooled bacterial suspension were added to each. To one were added 2 ml. of distilled water (3). The other replaced tube 2. Both were kept in ice whilst nitrogen was passed for $\frac{1}{2}$–$\frac{3}{4}$ hour to remove air. Then into tube 2 were run 2 ml. of leucomethylene blue from the burette, the ice was replaced by water at 40°, and the times for complete reduction in the two tubes were observed.

It will be seen (Table II) that the addition of the leuco-dye has no effect on the reduction. The acceleration noted above must therefore be due to the interaction of bacteria and substrate.

Table II.

| | Reduction time, mins. | |
|---|---|---|
| Dehydrogenase | Without leucomethylene blue | With leucomethylene blue |
| Formic | 35 | 36 |
| Glucose | 27 | 28 |
| Succinic | 55 | 54 |

## Discussion.

It has been seen that there is an accelerated reduction due to the formation of products from the substrate which can act as further hydrogen donators. What these products are in the case of formic acid it is difficult to know. One possibility would be the production of molecular hydrogen, which is known to act as a hydrogen donator with *Bact. coli* [Stephenson and Stickland, 1931]. But *Bact. coli*, grown as in this work in Roux bottles on caseinogen broth, is unable to liberate measurable quantities of hydrogen from formate [Yudkin, 1932]. It has also been shown that, in disagreement with the results with glucose and succinate, the effect of leaving bacteria in the presence of formate does not give a definite acceleration. There seem to be two possibilities. The first is that very small quantities of hydrogen are indeed produced, sufficient to reduce part of the methylene blue, but not sufficient to be demonstrated in the technique used for determining the amount of hydrogenlyase [Yudkin, 1932]. The second possibility is that in the presence of methylene blue products are formed from the formate which are different from those formed in its absence. Again, what such products are is difficult to imagine.

## Summary.

The several factors involved in determining the "reduction time" of methylene blue are well brought out by a study of the rate of reduction of the dye. Other phenomena, such as the difference in behaviour of formic dehydrogenase and succinic dehydrogenase in reducing varying quantities of methylene blue are also readily explained by these factors as shown by following the rate of reduction.

It has been shown that the presence of leucomethylene blue does not influence the reduction time of the dehydrogenases of glucose, formic acid or succinic acid.

## REFERENCES.

Ambrus, Banga and Szent-Györgyi (1931). *Biochem. Z.* **240**, 473.
Cannan, Cohen and Clark (1926). *U.S. Pub. Health. Reps.* Suppl. 55.
Quastel and Whetham (1924). *Biochem. J.* **18**, 519.
Stephenson and Stickland (1931). *Biochem. J.* **25**, 205.
Yudkin (1932). *Biochem. J.* **26**, 1859.
—— (1933). *Biochem. J.* **27**, 1849.

# CXCV. THE DEHYDROGENASES OF *BACTERIUM COLI*.

## III. THE COENZYME OF GLUCOSE DEHYDROGENASE.

By JOHN YUDKIN[1].

*From the Department of Colloid Science and the Biochemical Laboratory, Cambridge.*

(*Received June 30th, 1934.*)

The presence of a coenzyme of glucose dehydrogenase has been demonstrated in *Bact. coli* [Yudkin, 1933]. It was shown that the reduction of methylene blue by a dilute suspension of bacteria in the presence of glucose is much more rapid if a heated preparation of the bacteria is added. The properties and possible identity of the coenzyme are discussed in the present paper. The bacteria were prepared as described in the previous paper [Yudkin, 1933]. The preparation was usually diluted so as to give a reduction time of more than 30 minutes when no coenzyme was added. As standard coenzyme preparation, a suspension of bacteria about twice as strong was heated to 100° for 10–15 minutes. The coenzyme activity of a preparation was tested by comparing the acceleration produced in methylene blue reduction with that produced by a standard coenzyme preparation.

### 1. *Some properties of the coenzyme.*

The coenzyme is very stable to heat, acid and alkali. Bacteria heated for $6\frac{1}{2}$ hours at 100° showed no loss in coenzyme activity. After heating for another half hour to 120° in an autoclave, it was still active.

The following treatments with acid or alkali did not completely destroy coenzyme activity:

1. The preparation after being heated at 100° for $6\frac{1}{2}$ hours and then autoclaved for half an hour was made $N/500$ with $H_2SO_4$ or NaOH, heated to 100° for 10 minutes, cooled, neutralised and tested for activity.

2. A fresh preparation was made $N/50$ with $H_2SO_4$ or NaOH, heated in the autoclave to 120° for 20 minutes, left in the acid or alkali for 3 days, neutralised and tested.

3. A fresh preparation was made $N/20$ with $H_2SO_4$ or NaOH, heated to 100° for 5 minutes, cooled, neutralised and tested.

4. A fresh preparation was made $N/2$ with $H_2SO_4$ or NaOH, heated to 100° for 10 minutes, cooled, neutralised and tested.

In the experiments just described, fairly strong suspensions of bacteria were used, more than was necessary to give maximum activation. A partial destruction of the coenzyme would therefore not necessarily be shown. In order to demonstrate partial destruction, a standard preparation of coenzyme was made by heating bacteria to 100° for half an hour in water and diluting so as to give sub-maximum activation. Portions of the same preparation, after treatment with acid or alkali, were diluted to a known extent, and the activity was compared with that of the standard preparation. By this means it was shown that

[1] Benn W. Levy Student.

treatment with $N$ $H_2SO_4$ or NaOH at 100° for 40 minutes or at 120° for 20 minutes results in more than half the coenzyme being destroyed (Table XIII, p. 1469).

### 2. *Extraction of the coenzyme from* Bact. coli.

Before attempting to extract the coenzyme, some bacteria were ashed in order to see if the factor was purely inorganic, such as pyrophosphate or magnesium ions. It was found that bacterial ash caused an inhibition of the activity of the glucose dehydrogenase.

The coenzyme cannot be filtered through a bacterial filter. Two types of filter were used—a Seitz filter, made of nickel, and a metal-free porcelain filter, but the filtrates through both always caused an inhibition.

On the other hand, part of the coenzyme remains in the supernatant fluid after centrifuging down the bacteria. Both the clear supernatant fluid and the bacteria activate the glucose dehydrogenase. Repeated centrifuging with fresh quantities of distilled water gradually washes the coenzyme out of the bacteria (Table I).

Table I. *Washing out of coenzyme by water.*

Bacteria heated to 100° for 10 mins.

| | Reduction time, mins. |
|---|---|
| Bacteria alone | 67 |
| Bacteria *plus* 1st centrifugate | 34 |
| ,, ,, 4th centrifugate | 70 |
| ,, ,, suspension after 5th centrifuging | 30 |
| ,, ,, suspension after 8th centrifuging | 38 |

Heating the bacteria for a longer time gives an increased amount of coenzyme in the centrifugate (Table II).

Table II. *Extraction of coenzyme by heating.*

| | Reduction time, mins. |
|---|---|
| Bacteria alone | 95 |
| Bacteria *plus* standard coenzyme preparation | 32 |
| ,, ,, centrifugate after heating 5 mins. | 45 |
| ,, ,, centrifugate after heating 3 hours | 30 |

The centrifugate from fresh bacteria, which had been standing a short while at room temperature, had a small coenzymic activity (Table III).

Table III. *Washing out of coenzyme from fresh bacteria.*

| | Reduction time, mins. |
|---|---|
| Bacteria alone | 31 |
| Bacteria *plus* standard coenzyme preparation | 16 |
| ,, ,, centrifugate from fresh bacteria | 25 |

The coenzyme is removed from the centrifugate by filtration through a bacterial filter. Drying the centrifugate at 100° does not destroy its activity. Ashing it, however, results in its complete destruction—this ash, as that of the whole bacteria, gives an inhibition.

The coenzyme may be dialysed out of the preparations through a collodion membrane. The dialysis proceeds fairly slowly. After 3 days of dialysis against a large volume of distilled water changed every 12 hours, there is still a slight activity left in the bacterial preparation.

3. *Some properties of glucose dehydrogenase in relation to the coenzyme.*

It was thought that the well-known inhibitory effect of toluene on the enzyme might be due to a destruction of the coenzyme. That this is not so is shown by the fact that bacteria treated with toluene can act as a coenzyme preparation (Table IV).

Table IV. *Fresh bacteria treated with toluene 3 minutes. Excess toluene removed by passing air.*

| | Reduction time, mins. |
|---|---|
| Bacteria alone | 31 |
| Bacteria *plus* toluene-treated bacteria | 16 |
| Toluene-treated bacteria alone | ∞ |

It has often been observed that the glucose dehydrogenase of *Bact. coli* loses its activity more rapidly when left in dilute suspension than in more concentrated suspension. A possible explanation of this is that the coenzyme is removed more completely in the dilute suspension. This was tested as follows. Part of a concentrated suspension was diluted 1 in 50 and left together with some of the undiluted suspension for 2 days in the refrigerator. After this time, the concentrated suspension was also diluted 1 in 50, and both were tested for glucose dehydrogenase activity, with and without coenzyme. The coenzyme preparation was sufficient to give maximum acceleration. It is seen (Table V) that the loss of activity is not completely restored by addition of coenzyme and must therefore be due to an inactivation of the enzyme itself.

Table V.

| | Reduction time, mins. |
|---|---|
| Bacteria freshly diluted | 95 |
| ,, *plus* coenzyme | 21 |
| Bacteria diluted 48 hours previously | 150 |
| ,, ,, *plus* coenzyme | 34 |

4. *Replacement of the coenzyme by other factors.*

The adenylic acids, alone or in combination with pyrophosphate, have been shown to be the coenzymes of several reactions in which glucose is involved, such as the fermentation of yeast [Euler and co-workers: see Myrbäck, 1933] muscle glycolysis (Meyerhof and co-workers) and the dehydrogenation of glucose by the enzyme from liver (Mann, private communication; also Andersson [1934]). The dehydrogenation of lactic acid by an enzyme from heart-muscle involves a coenzyme, which is supposed to be identical with yeast cozymase [Banga and Szent-Györgyi, 1933; Andersson, 1934]. Yeast cozymase is also able to activate several dehydrogenations by enzymes from yeast and cucumber seed [Andersson, 1933]. The fermentations induced by lactic acid and propionic acid bacteria involve a coenzyme, which is probably also cozymase [Virtanen, 1925, 1, 2].

A number of coenzyme preparations from different sources have been tried in an attempt to replace the coenzyme of *Bact. coli*.

Preparations of the lactic coenzyme prepared from heart-muscle were unsuitable owing to the fact that they contained hydrogen donators which caused a rapid reduction of the methylene blue by *Bact. coli* even in the absence of glucose. A preparation of lactic acid coenzyme, made from yeast, caused an inhibition of the bacterial glucose dehydrogenase.

Muscle "Kochsaft" could not be used, since this too contained hydrogen donators. The coenzyme of lactic acid formation in muscle, adenylpyrophos-

phate, was prepared, and although active with muscle, was found also to cause an inhibition with bacteria.

A preparation of muscle-adenylic acid, however, is able to replace the glucose dehydrogenase coenzyme of *Bact. coli*, but it is far less efficient than heated bacteria. As much as 5 mg. are needed in each Thunberg tube in order to give an activation approaching that of a coenzyme preparation containing 0·3 mg. dry weight of bacteria (Table VI).

Table VI. *Muscle-adenylic acid as coenzyme.*

| | Reduction time, mins. |
|---|---|
| Bacteria alone | 42 |
| Bacteria *plus* 0·5 mg. adenylic acid | 40 |
| ,, ,, 2·5 mg. adenylic acid | 35 |
| ,, ,, 5 mg. adenylic acid | 22 |
| ,, ,, coenzyme preparation (0·3 mg. dry weight) | 18 |

Adenylic acid itself acts as a feeble hydrogen donator in the presence of methylene blue.

Since magnesium as well as the adenylic acid cozymase is necessary in yeast fermentation [Lohmann, 1931, 1] the effect of magnesium was tried with bacteria, too. It was found that 1 mg. magnesium gives an inhibition both with and without adenylic acid.

This partial replacement of the coenzyme by muscle-adenylic acid recalls the partial replacement of yeast cozymase by muscle-adenylic acid and *vice versa* [see Lohmann, 1931, 2, 3]. It suggests the possibility that the coenzyme is also related in constitution to adenylic acid. This observation is supported by the observation that the reduction time when both adenylic acid and coenzyme are present is intermediate between the reduction times with either alone (Table VII).

Table VII. *Effect of adenylic acid and coenzyme together.*

| | Reduction time, mins. |
|---|---|
| Bacteria alone | 200 |
| Bacteria *plus* coenzyme | 36 |
| ,, ,, adenylic acid (5 mg.) | 120 |
| ,, ,, adenylic acid and coenzyme | 48 |

This suggests the adsorption of both types of adenylic acid molecules at the enzyme, the adsorption of the muscle-adenylic acid hindering the adsorption of the more efficient coenzyme molecules.

A preparation of yeast cozymase (ACo between 10,000 and 20,000) was next tried and was found to replace the coenzyme completely in a concentration of less than 0·1 mg. in each tube. It seems therefore probable that the coenzyme of *Bact. coli* is identical with that of alcoholic fermentation by yeast. The experiments to be described are consistent with this assumption.

It was suggested (Mann, private communication) that the coenzyme of liver glucose dehydrogenase is identical with cozymase. This is supported by the experiments of Andersson [1934]. A preparation of the liver coenzyme [Harrison, 1933] was found to replace the coenzyme of *Bact. coli* (Table VIII).

Table VIII. *Coenzyme of liver glucose dehydrogenase as bacterial coenzyme.*

40 mg. of the preparation of liver coenzyme were used in each tube.

| | Reduction time, mins. |
|---|---|
| Bacteria alone | 46 |
| Bacteria *plus* coenzyme (heated bacteria) | 18 |
| ,, ,, liver coenzyme | 18 |

Not only can the liver coenzyme replace the bacterial coenzyme in the glucose dehydrogenation by bacteria, but the bacterial coenzyme can replace the former in liver enzyme preparations. That is, heated bacteria accelerate the reduction by liver glucose dehydrogenase to the same extent as the liver coenzyme itself (Table IX).

Table IX. *Bacterial coenzyme as liver coenzyme.*

47 mg. liver enzyme preparation in each tube.

| | Reduction time, mins. |
|---|---|
| Liver enzyme alone | 22½ |
| Liver enzyme *plus* liver coenzyme | 8¼ |
| ,, ,, coenzyme (heated bacteria) | 8 |

This lends strong support to the view that the glucose dehydrogenase of *Bact. coli* and of liver are the same and are both identical with yeast cozymase.

The system which oxidises hexosemonophosphate [Warburg and Christian, 1931] has been shown to involve a coenzyme, which more recently was found to consist of more than one factor [Warburg and Christian, 1933]. The crude coenzyme preparation from horse-blood is able to replace the bacterial coenzyme (Table X). It will be seen that the preparation of Warburg's coenzyme itself contains substances capable of acting slightly as hydrogen donators to methylene blue in the presence of *Bact. coli*. This accounts for the time of reduction being less with the Warburg coenzyme (16 minutes) than with the standard coenzyme preparation.

Table X. *Warburg's coenzyme as bacterial coenzyme.*

50 mg. crude coenzyme in each tube.

| | Reduction time, mins. |
|---|---|
| Bacteria alone | 46 |
| Bacteria *plus* coenzyme (heated bacteria) | 18 |
| ,, ,, Warburg's coenzyme | 16 |
| ,, ,, Warburg's coenzyme (without glucose) | 100 |
| Bacteria alone (without glucose) | >120 |

The interchanging of the coenzymes was further studied, and it was shown that although Warburg's coenzyme can replace the liver coenzyme, the latter cannot replace the former. Liver enzyme with Warburg's coenzyme gave a reduction time of 7 minutes (compare Table IX). These results suggest that the complex which we have described as Warburg's coenzyme contains cozymase and, in addition, other essential factors which are not present in the liver coenzyme preparations.

5. *Presence of cozymase in preparations of the coenzyme.*

It has been shown that cozymase is able to replace the coenzyme in *Bact. coli*. The converse is also true. Heated suspensions of the bacteria are able to reactivate washed yeast (apozymase).

The fermentations were measured in the Barcroft manometer at 30°. A $M/10$ solution of sodium hexosediphosphate was made in $M/20$ phosphate buffer, $p_H$ 6·5, containing 10 % glucose. To 0·2 g. of apozymase in the left-hand cup were added 1 ml. of this hexosediphosphate solution and 1 ml. of water or the solution to be tested for cozymase activity. The right-hand cup contained 2 ml. of water. In these conditions, the apozymase preparation with no cozymase added showed no measurable output of $CO_2$ in the course of the experiments (about 1 hour).

In order to obtain thick suspensions of bacteria, they were grown on tryptic caseinogen broth containing 0·5 % glucose and aerated during growth. The washed suspensions were heated for 15 minutes before determining the cozymase activity. Three other species of bacteria which had been found to contain the coenzyme of the glucose dehydrogenase of *Bact. coli* (see below) were also tested, as well as a preparation of dried baker's yeast and the cozymase preparation used in the experiments described previously. The results are expressed in ml. of $CO_2$ per hour per g. dry weight of preparation (Table XI). The dry weight of the bacteria was calculated after determination of the nitrogen content of the suspensions [Yudkin, 1932].

The cozymase activities of the preparation are thus expressed in the same form as by Euler and Myrbäck [see Myrbäck, 1933]. The conditions used in these experiments were, except for the use of the Barcroft manometers, the same as those used by these authors. The values, however, are about one-tenth those expected. For example, dried yeast should have ACo about 80–90. But in spite of this, the relative concentrations of cozymase in the different preparation can be seen from the values given.

Table XI.

| Preparation | Dry weight g. | $CO_2$ ml. per hour | $CO_2$ ml. per hour per g. |
|---|---|---|---|
| *Bact. coli* | 0·033 | 0·875 | 26·5 |
| *Bact. lactis aerogenes* (strain 124) | 0·071 | 2·34 | 33 |
| *Bact. alkaligenes* | 0·051 | 1·70 | 33 |
| *Bact. dispar* | 0·035 | 1·90 | 54 |
| Dried yeast | 0·200 | 1·62 | 8 |
| Cozymase | 0·001 | 1·16 | 1160 |

Cozymase is known to occur in other bacteria, *e.g.* in *Streptococcus lactis* [Myrbäck and Euler, 1924, 1] and in *B. casei* and propionic acid bacteria [Virtanen, 1925, 1, 2]. Myrbäck and Euler failed to find any cozymase in *Bact. lactis aerogenes* and they deduce from this that the initial splitting of the glucose molecule is different from that produced by *Streptococcus lactis*, or by baker's or Torula yeasts. Such deductions are somewhat hazardous. For example, *Bact. alkaligenes*, although it possesses no glucose dehydrogenase [Quastel and Wooldridge, 1925] and does not ferment glucose at all, is active both as glucose dehydrogenase coenzyme (see below) and cozymase. The negative results of Myrbäck and Euler may be due to their having used a different strain of the organism, but may also be due to the fact that these authors used a preparation of bacteria dried in air and not a heated suspension. The cells of *Bact. lactis aerogenes* are surrounded by a thick capsule which is probably not destroyed by drying but is by heating, thus liberating the cozymase.

*Relation between cozymase and coenzyme activities.* It has been shown that the coenzyme is fairly stable to heating in acid or alkali. Yeast cozymase appears to be less stable, particularly when purified [Myrbäck and Euler, 1924, 2], and it might have been expected that it would be completely destroyed in some of the experiments described above. However, in all cases where coenzyme activity persists after treatment, cozymase activity can also be demonstrated, although often greatly diminished (Table XII).

It will be seen that the treatments in Exp. 3 decrease the activity to 1/4 or less. Estimation of the glucose dehydrogenase coenzyme in the same suspensions shows that at least more than half the activity is lost. A was diluted so as to give sub-maximum activation (Table XIII).

Table XII. *Cozymase activity of* Bact. coli *after treatment with acid or alkali.*

| | | ml. $CO_2$ per hour |
|---|---|---|
| Exp. 1. | Heated bacteria | 650 |
| | After 10 mins. in $N/30$ $H_2SO_4$ at 100° | 425 |
| | „ 10 „ $N/30$ NaOH „ | 135 |
| | „ 20 „ $N/30$ $H_2SO_4$ at 120° | 170 |
| | „ 20 „ $N/30$ NaOH „ | 150 |
| Exp. 2. | Heated bacteria | 580 |
| | After 20 mins. in $N/2$ HCl at 120° | 84 |
| | „ 20 „ $N/2$ NaOH „ | 125 |
| Exp. 3. | A. Heated bacteria | 310 |
| | B. After 40 mins. in $N$ $H_2SO_4$ at 100° | 72 |
| | C. „ 40 „ $N$ NaOH „ | 70 |
| | D. „ 20 „ $N$ $H_2SO_4$ „ | 75 |
| | E. „ 20 „ $N$ NaOH „ | 56 |

Table XIII. *Activities of coenzyme after treament with acid or alkali* (see Table XII).

| | Reduction time, mins. |
|---|---|
| Bacteria alone | >180 |
| Bacteria *plus* A (1 in 300) | 60 |
| „ „ B (1 in 150) | 100 |
| „ „ C (1 in 150) | 120 |
| „ „ D (1 in 150) | 120 |
| „ „ E (1 in 150) | 140 |

The coenzyme activity of B is rather greater than that of the others although its cozymase activity is the same as those of C and D. But these small differences, which have been observed in several other cases, may be due to the formation during the treatment of substances which inhibit either the dehydrogenase or the yeast fermentation. Inhibition of the dehydrogenase for example was observed in certain cases after alkali treatment, where even in fairly high concentrations, the preparation never gave more than a certain submaximum activation.

From these experiments, we are justified in concluding that the destruction of the cozymase activity of bacterial suspensions is more or less parallel with the destruction of the coenzyme.

### 6. *Presence of the glucose dehydrogenase coenzyme of* Bact. coli *in other bacteria.*

Three other species of bacteria have been tested for the presence of the coenzyme: *Bact. dispar*, *Bact. lactis aerogenes* and *Bact. alkaligenes*. They were grown in tryptic caseinogen broth, and preparations for the testing of coenzyme activity were made by heating washed suspensions, as with *Bact. coli*. All three species contain the coenzyme (Table XIV).

Table XIV. *Coenzyme of* Bact. coli *in other bacteria.*

| | | Reduction time, mins. |
|---|---|---|
| Exp. 1. | *Bact. coli* alone | 100 |
| | *Bact. coli* + heated *Bact. coli* | 26 |
| | „ + heated *Bact. alkaligenes* | 26·5 |
| Exp. 2. | *Bact. coli* alone | >180 |
| | *Bact. coli* + heated *Bact. coli* | 36 |
| | „ + heated *Bact. alkaligenes* | 34 |
| Exp. 3. | *Bact. coli* alone | 50 |
| | *Bact. coli* + heated *Bact. coli* | 27 |
| | „ + heated *Bact. dispar* | 28 |

7. *Coenzyme of glucose dehydrogenase of other bacteria.*

The abnormal falling off in activity of the glucose dehydrogenase on dilution, which suggested the presence of the coenzyme in *Bact. coli* [Yudkin, 1933], is also observed with *Bact. lactis aerogenes* and *Bact. dispar*.

An activation occurs with *Bact. dispar* on adding, to a dilute suspension of the bacteria, heated preparations of any of the four bacteria mentioned, or by cozymase (Table XV).

Table XV. *Glucose dehydrogenase coenzyme of* Bact. dispar.

| | Reduction time, mins. |
|---|---|
| *Bact. dispar* undiluted | 22 |
| ,, diluted 1 in 2 | 80 |
| ,, + heated *Bact. coli* | 33 |
| ,, + heated *Bact. lactis aerogenes* | 33 |
| ,, + heated *Bact. dispar* | 31·5 |
| ,, + 1 mg. cozymase | 31 |
| ,, + 0·5 mg. cozymase | 45 |

*Bact. dispar* requires more cozymase to reactivate its glucose dehydrogenase than either *Bact. coli* or *Bact. lactis aerogenes* (Table XVI). It is interesting to compare this with the relatively larger amount of cozymase present in the former (Table XI).

In the case of *Bact. lactis aerogenes*, activation does not occur on the addition of heated bacteria but only on the addition of cozymase. This is caused by inhibiting substances present in the heated preparations. That this effect is due to the glucose dehydrogenase of *Bact. lactis aerogenes* which is sensitive to these substances and not to differences in the nature of the heated bacteria is shown by the fact that heated *Bact. coli* also inhibit the enzyme of *Bact. lactis aerogenes* (Table XVI). On the other hand, heated *Bact. lactis aerogenes* do not cause an inhibition of the enzyme of *Bact. coli* (Table XIV) or of *Bact. dispar* (Table XV). Moreover, the activation of the enzyme of *Bact. lactis aerogenes* by cozymase is nullified by the presence of heated bacteria. A decrease in the concentration of the heated bacteria, while decreasing the amount of coenzyme, also decreases the concentration of the inhibitory substances, and a greater activity is observed.

Table XVI. *Glucose dehydrogenase coenzyme of* Bact. lactis aerogenes.

| | | Reduction time, mins. |
|---|---|---|
| Exp. 1. | *Bact. lactis aerogenes* undiluted | 14 |
| | ,, diluted 1 in 2 | 70 |
| Exp. 2. | *Bact. lactis aerogenes* alone | 16 |
| | ,, + boiled *Bact. lactis aerogenes* | 70 |
| | ,, + boiled *Bact. lactis aerogenes* (1 in 2) | 55 |
| | ,, + boiled *Bact. lactis aerogenes* (1 in 3) | 40 |
| | ,, + boiled *Bact. coli* | 71 |
| | ,, + boiled *Bact. coli* (1 in 2) | 49 |
| | ,, + cozymase (0·15 mg.) | 12 |
| Exp. 3. | *Bact. lactis aerogenes* alone | 32 |
| | ,, + boiled *Bact. lactis aerogenes* | 50 |
| | ,, + cozymase (0·2 mg.) | 21 |
| | ,, + boiled *Bact. lactis aerogenes* + cozymase (0·2 mg.) | 43 |

8. *Participation of the coenzyme in other reactions with glucose.*

(*a*) *The oxidation of glucose by oxygen.* The oxygen uptakes of the bacteria with and without coenzyme were measured in the Warburg manometer at 37°.

Five manometers were used; each contained 1 ml. of bacterial suspension, 1 ml. of $M/20$ phosphate buffer, $p_H$ 7·0, and in addition the following:

| Manometer | Glucose | Coenzyme | Cozymase (1 mg.) | Water |
|---|---|---|---|---|
| 1 | 1 | 0 | 0 | 2 |
| 2 | 0 | 0 | 0 | 3 |
| 3 | 1 | 1 | 0 | 1 |
| 4 | 0 | 1 | 0 | 2 |
| 5 | 1 | 0 | 1 | 1 |

The results are shown in Table XVII together with the reduction times of methylene blue by the same mixtures.

Table XVII.

| | Exp. 1 | | Exp. 2 | | Exp. 3 |
|---|---|---|---|---|---|
| Manometer containing | $O_2$ uptakes ml./hour | Reduction time mins. | $O_2$ uptakes ml./hour | Reduction time mins. | $O_2$ uptakes ml./hour |
| 1. Bacteria + glucose | 6 | 45 | 11·5 | 33 | 7 |
| 2. Bacteria alone | 3 | ∞ | 6·5 | ∞ | — |
| 3. Bacteria, glucose and coenzyme | 39 | 22 | 28·5 | 23 | 18 |
| 4. Bacteria and coenzyme | 11·5 | ∞ | 11·5 | ∞ | — |
| 5. Bacteria, glucose and cozymase | — | — | — | — | 17 |

The oxygen uptakes are seen to be greatly increased by the presence of coenzyme. The relative acceleration is much greater than for the reduction of methylene blue; *e.g.* in Exp. 1, the oxygen uptake is accelerated more than six times by the coenzyme, whereas the reduction time is only halved. It will be seen that there is a comparatively large uptake with bacteria and coenzyme without glucose (4) although there was no reduction at all of the methylene blue after 5 hours. This difference in behaviour between the oxidations by methylene blue and oxygen is not yet explained.

As with the methylene blue experiments, the coenzyme may be replaced by cozymase.

(*b*). *The liberation of hydrogen from glucose.* In order to obtain a suspension of bacteria containing the enzyme glucose hydrogenlyase capable of performing this reaction, *Bact. coli* was grown on a glucose broth medium in Roux bottles and the activity of the enzyme measured in the Barcroft manometer [Yudkin, 1932].

The action of glucose hydrogenlyase also involves a coenzyme which is present in heated bacteria and is replaceable by cozymase (Table XVIII).

Table XVIII. *Coenzyme of glucose hydrogenlyase.*

| | $H_2$, $\mu$l. per hour |
|---|---|
| Bacteria alone | 0 |
| ,, +heated bacteria | 25 |
| ,, +1 mg. cozymase | 15 |

The demonstration of the coenzyme with this enzyme is not so satisfactory as with the dehydrogenase, since when the bacteria have been diluted sufficiently to wash out the coenzyme, the activity even in the presence of coenzyme is very low.

## Discussion.

The results described above are all in agreement with the hypothesis that the glucose dehydrogenase coenzyme of *Bact. coli* is identical with cozymase. Moreover, they support the view of Andersson that the coenzyme of liver glucose dehydrogenase is also cozymase.



**Vol. XXVIII, page 1471, Table XVII**

***For*** **$O_2$ uptakes ml./hour** ***read*** **$O_2$ uptakes $\mu$l./hour**

It has been stated that the liver coenzyme is partially activated by adenylpyrophosphate from muscle [Harrison, 1933], whereas we have seen that the enzyme of *Bact. coli* is inhibited by this. This does not necessarily imply that the coenzymes cannot be the same. If the enzymes are different, one can imagine that both may be activated by the same substance which occurs naturally with them, but that a second substance might produce an activation with one and an inhibition with the other. The glucose dehydrogenases of liver and *Bact. coli* are certainly very different. For example, the affinity of the former for glucose is unusually low, the optimum concentration being 0·25 *M* [Harrison, 1931], whereas the affinity of the bacterial enzyme is so high that it cannot be measured.

SUMMARY.

1. It is suggested that the coenzyme involved in the dehydrogenation of glucose by *Bact. coli* is identical with the cozymase of yeast. The evidence for this is:

(*a*) Bacterial coenzyme may be replaced by very small concentrations of cozymase.

(*b*) Liver coenzyme, which is itself replaceable by cozymase, can replace the bacterial coenzyme. This supports the view of Andersson that the liver coenzyme is also identical with cozymase.

(*c*) The bacterial coenzyme can replace the liver coenzyme.

(*d*) Warburg's coenzyme, which can replace the liver coenzyme, can also replace the bacterial coenzyme.

(*e*) Preparations which contain the coenzyme also contain cozymase, and the destruction of one involves the destruction of the other.

2. Warburg's coenzyme appears to contain cozymase as one of its components.

3. The coenzyme of the glucose dehydrogenase is also contained in three other bacteria—*Bact. alkaligenes*, *Bact. dispar* and *Bact. lactis aerogenes*. The latter two species also need a coenzyme for the dehydrogenation of glucose, which is replaceable by cozymase.

4. All four species of bacteria examined contain cozymase.

5. A coenzyme, found in heated bacteria, and replaceable by cozymase, is involved in two other reactions in which glucose is attacked by *Bact. coli*. These are the oxidation of glucose by oxygen, and the production of hydrogen from glucose by the enzyme glucose hydrogenylase.

My thanks are due to the following members of the Biochemical Laboratory, Cambridge. Dr H. L. A. Tarr, in collaboration with whom the experiments with the liver and Warburg coenzymes were carried out; Mr P. J. G. Mann, for the preparations of cozymase and lactic acid coenzyme and Mrs D. M. Needham, for the preparation of adenylpyrophosphate and the testing of its activity. I have also to thank Prof. H. v. Euler, for the preparation of apozymase and Prof. E. K. Rideal, for his continued interest in the work.

REFERENCES.

Andersson (1933). *Z. physiol. Chem.* **217**, 186.
—— (1934). *Z. physiol. Chem.* **225**, 57.
Banga and Szent-Györgyi (1933). *Z. physiol. Chem.* **217**, 38.
Harrison (1931). *Biochem. J.* **25**, 1016.
—— (1933). *Biochem. J.* **27**, 382.
Lohmann (1931, 1). *Naturwiss.* **19**, 180.
—— (1931, 2). *Biochem. Z.* **237**, 445.
—— (1931, 3). *Biochem. Z.* **241**, 67.
Myrbäck (1933). Cozymase; Ergebnisse der Enzymforschung II. (Leipzig.)
—— and Euler (1924, 1). *Ber. deutsch. chem. Ges.* **57**, 1073.
—— —— (1924, 2). *Z. physiol. Chem.* **138**, 6.
Quastel and Wooldridge (1925). *Biochem. J.* **19**, 652.
Virtanen (1925, 1). *Z. physiol. Chem.* **143**, 71.
—— (1925, 2). *Ber. deutsch. chem. Ges.* **58**, 2441.
Warburg and Christian (1931). *Biochem. Z.* **242**, 206.
—— —— (1933). *Biochem. Z.* **266**, 377.
Yudkin (1932). *Biochem. J.* **26**, 1859.
—— (1933). *Biochem. J.* **27**, 1849.

# CXCVI. THE EFFECT OF OESTRONE ON NORMAL AND CASTRATED MALE RATS.

By VLADIMIR KORENCHEVSKY and MARJORIE DENNISON.

*From the Lister Institute, London.*

(*Received July 1st, 1934.*)

It is now established that male and female hormones are produced in both sexes. In the urine of man and non-pregnant woman they are often excreted in approximately equal amounts [Dingemanse, 1931; Womack and Koch, 1932; Freud, 1933]. Zondek [1934] obtained the largest amount of oestrin from the urine of stallions, in which he found 170,000 M.U. per litre; while he obtained 200 M.U. from the non-pregnant and 100,000 M.U. from the pregnant mare; and from 30 to 200 M.U. in sexually mature woman and 10,000 M.U. in pregnant woman. Zondek and Euler [1934] suggest that in the organism oestrin is produced in the gonads, in the placenta and probably in some other tissues, since Loewe *et al.* [1932] found small amounts of oestrin even in the urine of castrated animals. These facts make necessary a careful examination of the influence of oestrin on males.

That oestrin had a physiological influence on young castrated rats was shown by Freud [1933]. Both oestrone and oestriol promoted the development of smooth muscle in the seminal vesicles of such rats and increased the influence of testicular hormone on the seminal vesicles.

That oestrin has a pathological influence has also been demonstrated. Thus Moore and Price [1932] injected 10–15 rat units of oestrin for 20 days into adult male rats and obtained a marked decrease in size of the testes, seminal vesicles and prostate, the atrophy of these last two organs sometimes being equal to that in castrates. Simultaneous injection of gonad-stimulating, or testicular hormone, however, prevented the injurious effect of oestrin (except the testicular damage which was not prevented when testicular hormone was used). In castrated male rats no effect was produced by injections for 20 days of oestrin alone (12–40 R.U.), or in doses of 12 R.U. combined with 6 C.U. of testicular hormone.

Lacassagne [1933] used young, adult and castrated mice injected for different periods with about 500 M.U. per week of oestrin. During the first 2 weeks atrophy of the seminal vesicles and prostate were noted, but later a pathological enlargement of the posterior lobe of the prostate developed, with stratification and keratinisation of the epithelium. These changes were usually observed after treatment for 3 months.

Burrows and Kennaway [1934] painted the skin of mice twice a week, for 6–22 weeks, with a solution of oestrin in a dose of about 125 R.U. The following changes were obtained in male mice; scrotal hernia, atrophy of the testes and seminal vesicles, enlargement of the posterior lobe of the prostate with histological changes similar to those observed by Lacassagne, and even in some cases resembling those in tumours. These pathological changes indicate the need for caution in following the suggestion of Freud [1933] that oestrin should be used in clinical practice in males.

In the present paper, only the changes in weight of the sexual, endocrine and some other organs are given. The histological results will be published elsewhere.

Table I. *Age, average weight, gain in body weight, and period of injections of normal rats.*

| No. of litter | No. of rats in litter | Period of injections Days | Final age Days | Final weight, g. Controls | 20 u. | 60 u. | 180 u. | Gain in body weight, g. Controls | 20 u. | 60 u. | 180 u. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | 4 | 32 | 60 | 231 | 234 | — | — | 167 | 166 | — | — |
| 18 | 3 | 29 | 64 | 235 | 235 | — | — | 144 | 137 | — | — |
| 19 | 4 | 43 | 66 | 260 | — | 243 | — | 218 | — | 200 | — |
| 20 | 6 | 34 | 59 | 241 | — | 213 | — | 190 | — | 165 | — |
| 21 | 5 | 35 | 58 | 234 | — | 181 | — | 190 | — | 139 | — |
| 22 | 3 | 32 | 57 | 198 | — | — | 175 | 144 | — | — | 120 |
| 23 | 4 | 43 | 65 | 300 | — | — | 252 | 256 | — | — | 208 |
| 24 | 5 | 34 | 65 | 271 | — | — | 217 | 195 | — | — | 136 |
| 25 | 5 | 35 | 58 | 265 | — | — | 209 | 215 | — | — | 159 |

Table II. *Effect on sexual organs and thymus of prolonged injections of oestrone into normal rats.*

| | | Weights: Actual 20 u. | Actual 60 u. | Actual 180 u. | Per 200 g. body weight 20 u. | Per 200 g. body weight 60 u. | Per 200 g. body weight 180 u. |
|---|---|---|---|---|---|---|---|
| *Prostate with sem. ves.*, mg. | Controls | 951 | 1443 | 1326 | 817 | 1185 | 1015 |
| | Injected | 1015 | 658 | 633 | 865 | 603 | 568 |
| % change | Average | — | **−54** | **−55** | — | **−48** | **−45** |
| | Maximum variations | +17 | −71 | −72 | +16 | −65 | −67 |
| | | − 4 | −39 | −43 | − 5 | −31 | −32 |
| *Prostate*, mg. | Controls | 513 | 809 | 895 | 441 | 660 | 552 |
| | Injected | 515 | 417 | 413 | 460 | 384 | 373 |
| % change | Average | — | **−48** | **−45** | +5 | **−41** | **−33** |
| | Maximum variations | +2 | −67 | −60 | +9 | −57 | −54 |
| | | −1 | −35 | −32 | +1 | −26 | −19 |
| *Sem. ves.*, mg. | Controls | 439 | 648 | 606 | 377 | 525 | 463 |
| | Injected | 471 | 241 | 221 | 400 | 218 | 197 |
| % change | Average | — | **−62** | **−66** | — | **−57** | **−59** |
| | Maximum variations | +26 | −79 | −86 | +26 | −74 | −83 |
| | | −13 | −46 | −56 | −14 | −39 | −48 |
| *Penis*, mg. | Controls | 336 | 310 | 309 | 289 | 253 | 246 |
| | Injected | 334 | 229 | 221 | 285 | 215 | 204 |
| % change | Average | — | **−26** | **−28** | — | **−15** | — |
| | Maximum variations | −4 | −36 | −53 | −6 | −17 | −46 |
| | | +3 | −20 | −14 | +3 | −10 | + 2 |
| *Preputial glands*, mg. | Controls | 140 | 153 | 136 | 120 | 125 | 112 |
| | Injected | 146 | 117 | 107 | 125 | 111 | 101 |
| % change | Average | — | **−23** | **−24** | — | **−11** | — |
| | Maximum variations | +29 | −37 | −40 | +29 | −18 | −31 |
| | | −12 | −13 | −14 | −11 | − 3 | + 8 |
| *Testes*, mg. | Controls | 2765 | 2351 | 2482 | 2381 | 1918 | 1966 |
| | Injected | 2601 | 1973 | 2140 | 2220 | 1855 | 2031 |
| % change | Average | **−6** | **−16** | **−14** | **−7** | — | — |
| | Maximum variations | −8 | −23 | −34 | −6 | −10 | +25 |
| | | −4 | −10 | − 2 | −7 | + 2 | −24 |
| *Thymus*, mg. | Controls | 478 | 440 | 527 | 418 | 362 | 411 |
| | Injected | 516 | 518 | 431 | 441 | 493 | 403 |
| % change | Average | **+8** | — | **−17** | **+6** | **+40** | — |
| | Maximum variations | +9 | +54 | −30 | +7 | +67 | −14 |
| | | +7 | − 7 | − 7 | +5 | +20 | + 8 |

## Technique.

Experiments were performed on 39 normal rats belonging to 9 litters and on 73 castrated rats belonging to 16 litters. The number of rats in each litter, period of injection and daily dose of oestrin, the final age and weight of the rats and, in the case of castrated rats, the age at castration and the period after castration when killed, are given in Tables I and IV. In litters 14 and 15, control rats had very slight pneumonia and therefore were not excluded from the experiments, although there was some depressing effect on gain in body weight and fat deposition.

Table III. *Effect on adrenals, hypophysis, liver, kidneys, spleen, heart and food intake of prolonged injections of oestrone in normal rats.*

| | | Weights | | | | | |
|---|---|---|---|---|---|---|---|
| | | Actual | | | Per 200 g. body weight | | |
| | | 20 u. | 60 u. | 180 u. | 20 u. | 60 u. | 180 u. |
| *Adrenals*, mg. | Controls | 61·6 | 49·8 | 50·6 | 53·2 | 40·7 | 40·5 |
| | Injected | 75·0 | 67·1 | 72·7 | 64·1 | 62·1 | 68·1 |
| % change | Average | +22 | +34 | +45 | +20 | +53 | +73 |
| | Maximum | +27 | +57 | +60 | +24 | +61 | +94 |
| | variations | +16 | +18 | +32 | +17 | +44 | +42 |
| *Hypophysis*, mg. | Controls | 7·7 | 10·5 | 10·4 | 6·7 | 8·6 | 8·2 |
| | Injected | 9·2 | 11·5 | 13·0 | 7·9 | 11·1 | 12·2 |
| % change | Average | +20 | +12 | — | +19 | +29 | +52 |
| | Maximum | +29 | +32 | +46 | +29 | +41 | +75 |
| | variations | +12 | + 1 | − 8 | + 9 | +16 | + 5 |
| *Liver*, g. | Controls | 15·65 | 9·66 | 11·84 | 13·33 | 7·96 | 9·44 |
| | Injected | 16·46 | 9·06 | 10·11 | 14·04 | 8·56 | 9·62 |
| % change | Average | +5 | — | −15 | +5 | + 8 | — |
| | Maximum | + 8 | −16 | −22 | +8 | +11 | +8 |
| | variations | + 2 | + 0·2 | −12 | +3 | + 6 | −1 |
| *Kidneys*, g. | Controls | 2·36 | 1·94 | 2·18 | 2·02 | 1·58 | 1·71 |
| | Injected | 2·33 | 1·76 | 1·89 | 1·99 | 1·66 | 1·79 |
| % change | Average | — | −10 | −13 | — | — | — |
| | Maximum | − 8 | −16 | −21 | −9 | +8 | +8 |
| | variations | +6 | − 5 | − 5 | +6 | −1 | −1 |
| *Spleen*, mg. | Controls | 581 | 529 | 566 | 497 | 434 | 440 |
| | Injected | 713 | 595 | 531 | 608 | 564 | 500 |
| % change | Average | +23 | +12 | — | +23 | +30 | — |
| | Maximum | +29 | +16 | −24 | +29 | +42 | +27 |
| | variations | +17 | + 9 | +12 | +17 | +23 | − 8 |
| *Heart*, mg. | Controls | 816 | 759 | 812 | 704 | 671 | 637 |
| | Injected | 823 | 728 | 760 | 702 | 692 | 714 |
| % change | Average | — | — | − 7 | — | — | +13 |
| | Maximum | + 2 | −13 | −11 | −3 | +12 | +21 |
| | variations | −0·4 | + 7 | − 3 | +2 | − 9 | + 7 |
| *Food intake*, g. | Controls | — | 50·3 | 54·4 | — | 48·8 | 45·2 |
| | Injected | — | 44·9 | 48·4 | — | 50·4 | 48·2 |
| % change | Average | — | −11 | −11 | — | +3 | +7 |
| | Maximum | — | −17 | −13 | — | +7 | +9 |
| | variations | — | − 6 | − 8 | — | +0·4 | +5 |

All the experiments were performed using the same technique as has been described in our previous papers. Oestrone, dissolved in olive oil after preliminary solution in a small quantity of the monoethyl ether of ethyleneglycol, was injected subcutaneously once a day. The pure crystalline preparation of oestrone was received from the Department of Biological Standards to which we wish to express our thanks. For litters 10, 12, 14 and 15 pure oestrone dissolved in arachis oil was kindly given to us by Dr A. S. Parkes, whom we also wish to thank.

Table IV. *Age, average weight and period of injections of castrated rats.*

| No. of litter | No. of rats in litter | Age in days: At castration | Age in days: When killed | No. of days after castration when killed | Final weight, g.: Control | 10 u. | 20 u. | 60 u. | 180 u. | Period of injections Days |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 4 | 23 | 74 | 51 | 275 | 270 | — | — | — | 7 |
| 2 | 3 | 28 | 64 | 36 | 200 | 228 | — | — | — | 7 |
| 3 | 4 | 23 | 74 | 51 | 243 | — | 264 | — | — | 7 |
| 4* | 2 | 23 | 74 | 51 | — | — | 266 | — | — | 7 |
| 5 | 4 | 21 | 72 | 51 | 267 | — | — | 254 | — | 7 |
| 6 | 2 | 27 | 63 | 36 | 208 | — | — | 176 | — | 7 |
| 7 | 6 | 25 | 65 | 40 | 245 | — | — | 244 | 233 | 7 |
| 8 | 3 | 21 | 72 | 51 | 268 | — | — | — | 244 | 7 |
| 9 | 4 | 25 | 61 | 36 | 205 | — | — | — | 192 | 7 |
| 10 | 4 | 26 | 106 | 80 | 285 | — | 303 | — | — | 29 |
| 11 | 3 | 23 | 78 | 55 | 340 | — | 265 | — | — | 43 |
| 12 | 4 | 25 | 104 | 79 | 284 | — | — | 277 | — | 28 |
| 13 | 3 | 23 | 78 | 55 | 289 | — | — | 252 | — | 43 |
| 14 | 3 | 28 | 93 | 65 | 261 | — | — | — | 294 | 28 |
| 15 | 3 | 23 | 88 | 65 | 281 | — | — | — | 291 | 28 |
| 16 | 3 | 22 | 77 | 55 | 380 | — | — | — | 270 | 43 |

* Control rat in litter 4 excluded because of severe pneumonia. In the following tables for litter 4 respective figures of control rats of the litter 3 are used, both litters being of the same age and castrated at the same date.

Table V. *Effect on sexual organs of 7 days of injections of oestrone into castrated rats.*

| | | Weights: Actual: 10 u. | 20 u. | 60 u. | 180 u. | Per 200 g. body weight: 10 u. | 20 u. | 60 u. | 180 u. |
|---|---|---|---|---|---|---|---|---|---|
| *Prostate with sem. ves.*, mg. | Controls | 72 | 72 | 70 | 70 | 62 | 60 | 59 | 58 |
| | Injected | 79 | 90 | 94 | 99 | 65 | 68 | 86 | 89 |
| % change | Average | **+10** | **+24** | **+34** | **+43** | **+5** | **+13** | **+45** | **+55** |
| | Maximum | +18 | +29 | +42 | +56 | +5 | +17 | +57 | +64 |
| | variations | + 3 | +19 | +28 | +29 | +5 | + 8 | +35 | +42 |
| *Prostate*, mg. | Controls | 61 | 58 | 58 | 57 | 52 | 48 | 49 | 48 |
| | Injected | 64 | 72 | 71 | 73 | 52 | 54 | 65 | 66 |
| % change | Average | — | **+23** | **+23** | **+30** | — | **+13** | **+31** | **+38** |
| | Maximum | +14 | +28 | +42 | +44 | +2 | +17 | +43 | +49 |
| | variations | − 5 | +19 | +14 | +16 | −2 | + 8 | +19 | +26 |
| *Sem. ves.*, mg. | Controls | 12 | 15 | 14 | 13 | 10 | 12 | 10 | 10 |
| | Injected | 16 | 18 | 23 | 26 | 13 | 14 | 22 | 23 |
| % change | Average | **+37** | **+20** | **+ 89** | **+102** | **+37** | **+17** | **+110** | **+105** |
| | Maximum | +44 | +27 | +115 | +122 | +40 | +25 | +166 | +140 |
| | variations | +29 | +13 | + 50 | + 75 | +33 | + 8 | + 51 | + 82 |
| *Penis*, mg. | Controls | 91 | 100 | 96 | 94 | 76 | 82 | 84 | 79 |
| | Injected | 109 | 96 | 97 | 104 | 88 | 73 | 87 | 94 |
| % change | Average | **+20** | — | — | **+10** | **+14** | **−12** | — | **+20** |
| | Maximum | +26 | −10 | −20 | +18 | +16 | −17 | +24 | +30 |
| | variations | +15 | + 2 | +16 | + 5 | +11 | − 6 | − 1 | +12 |
| *Preputial glands*, mg. | Controls | 63 | 71 | 57 | 59 | 54 | 59 | 49 | 48 |
| | Injected | 70 | 68 | 65 | 80 | 55 | 51 | 63 | 75 |
| % change | Average | — | **−5** | **+13** | **+39** | — | **−14** | **+27** | **+58** |
| | Maximum | +41 | −6 | +20 | +49 | +44 | −14 | +41 | +73 |
| | variations | −22 | −4 | + 7 | +30 | −32 | −14 | +14 | +42 |

Table VI. *Effect on sexual organs and hypophysis of prolonged injections of oestrone into castrated rats.*

| | | Weights: Actual | | | Weights: Per 200 g. body weight | | |
|---|---|---|---|---|---|---|---|
| | | 20 u. | 60 u. | 180 u. | 20 u. | 60 u. | 180 u. |
| *Prostate with sem. ves.*, mg. | Controls | 80 | 74 | 80 | 52 | 52 | 52 |
| | Injected | 96 | 123 | 148 | 69 | 95 | 101 |
| % change | Average | **+20** | **+68** | **+ 89** | **+33** | **+ 85** | **+100** |
| | Maximum | +20 | +90 | +110 | +53 | +102 | +121 |
| | variations | +19 | +46 | + 57 | +13 | + 68 | + 84 |
| *Prostate*, mg. | Controls | 63 | 60 | 65 | 41 | 42 | 43 |
| | Injected | 68 | 85 | 92 | 48 | 66 | 64 |
| % change | Average | **+ 8** | **+45** | **+44** | **+20** | **+59** | **+50** |
| | Maximum | +10 | +66 | +66 | +41 | +74 | +52 |
| | variations | + 6 | +23 | + 9 | ± 0 | +43 | +46 |
| *Sem. ves.*, mg. | Controls | 18 | 15 | 12 | 12 | 10 | 12 |
| | Injected | 29 | 38 | 49 | 21 | 29 | 35 |
| % change | Average | **+66** | **+165** | **+322** | **+82** | **+198** | **+358** |
| | Maximum | +85 | +200 | +340 | +86 | +238 | +443 |
| | variations | +48 | +129 | +292 | +78 | +158 | +289 |
| *Penis*, mg. | Controls | 86 | 95 | 101 | 57 | 67 | 66 |
| | Injected | 100 | 98 | 107 | 71 | 75 | 75 |
| % change | Average | **+17** | — | — | **+31** | **+12** | **+16** |
| | Maximum | +23 | +12 | +45 | +57 | +15 | +41 |
| | variations | +11 | − 5 | −26 | + 4 | + 9 | + 3 |
| *Hypophysis*, mg. | Controls | 13·7 | 13·5 | 13·7 | 8·9 | 9·4 | 9·2 |
| | Injected | 15·3 | 17·9 | 21·3 | 10·9 | 13·8 | 14·9 |
| % change | Average | **+12** | **+33** | **+61** | **+24** | **+48** | **+63** |
| | Maximum | +13 | +45 | +95 | +44 | +54 | +72 |
| | variations | +11 | +22 | +19 | + 3 | +41 | +49 |

Table VII. *Effect on hypophysis, thymus, kidneys and heart of 7 days of injections of oestrone into castrated rats.*

| | | Weights: Actual | | | | Weights: Per 200 g. body weight | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 10 u. | 20 u. | 60 u. | 180 u. | 10 u. | 20 u. | 60 u. | 180 u. |
| *Hypophysis*, mg. | Controls | 11·5 | 12·4 | 11·9 | 11·4 | 9·9 | 10·3 | 10·0 | 9·6 |
| | Injected | 15·5 | 14·6 | 14·3 | 14·7 | 12·3 | 11·1 | 12·9 | 13·3 |
| % change | Average | **+35** | **+17** | **+22** | **+30** | **+26** | **+ 7** | **+30** | **+38** |
| | Maximum | +40 | +20 | +43 | +37 | +43 | +10 | +43 | +44 |
| | variations | +30 | +15 | ± 0 | +25 | +10 | + 5 | +18 | +34 |
| *Thymus*, mg. | Controls | 585 | 550 | 680 | 699 | 504 | 452 | 611 | 590 |
| | Injected | 547 | 697 | 583 | 515 | 449 | 525 | 540 | 475 |
| % change | Average | — | **+27** | **−21** | **−25** | **−12** | **+16** | — | — |
| | Maximum | −24 | +30 | −20 | −37 | −23 | +18 | −26 | −35 |
| | variations | +12 | +23 | − 8 | − 5 | − 2 | +14 | + 8 | + 3 |
| *Kidneys*, g. | Controls | 1·94 | 1·79 | 1·90 | 1·98 | 1·66 | 1·47 | 1·60 | 1·66 |
| | Injected | 2·10 | 2·05 | 2·14 | 2·15 | 1·70 | 1·55 | 1·92 | 1·94 |
| % change | Average | **+ 9** | **+15** | — | **+ 9** | **+3** | **+5** | **+20** | **+17** |
| | Maximum | +16 | +17 | +25 | +17 | +4 | +7 | +25 | +23 |
| | variations | + 1 | +12 | − 1 | + 1 | +2 | +4 | +17 | +11 |
| *Heart*, mg. | Controls | 725 | 682 | 705 | 694 | 617 | 562 | 588 | 582 |
| | Injected | 810 | 776 | 723 | 719 | 653 | 588 | 646 | 645 |
| % change | Average | **+12** | **+14** | — | — | **+ 6** | **+5** | **+10** | **+11** |
| | Maximum | +16 | +15 | +18 | + 8 | +11 | +6 | +18 | +14 |
| | variations | + 9 | +12 | − 3 | − 1 | + 1 | +3 | + 2 | + 5 |

Table VIII. *Effect on thymus, kidneys, spleen and heart of prolonged injections of oestrone into castrated rats.*

| | | Weights | | | | | |
|---|---|---|---|---|---|---|---|
| | | Actual | | | Per 200 g. body weight | | |
| | | 20 u. | 60 u. | 180 u. | 20 u. | 60 u. | 180 u. |
| *Thymus*, mg. | Controls | 681 | 492 | 704 | 425 | 344 | 451 |
| | Injected | 467 | 439 | 479 | 336 | 333 | 338 |
| % change | Average | **-27** | — | **-27** | **-21** | — | **-24** |
| | Maximum | -38 | -17 | -49 | -21 | -5 | -34 |
| | variations | -15 | +1 | -7 | -21 | +0·4 | -10 |
| *Kidneys*, g. | Controls | 2·06 | 1·98 | 2·38 | 1·32 | 1·38 | 1·55 |
| | Injected | 1·96 | 2·04 | 2·40 | 1·38 | 1·54 | 1·68 |
| % change | Average | — | — | — | **+4** | **+11** | **+8** |
| | Maximum | -15 | +13 | -23 | +9 | +16 | +9 |
| | variations | +7 | -8 | +22 | +0·1 | +7 | +8 |
| *Spleen*, mg. | Controls | 633 | 530 | 620 | 408 | 369 | 395 |
| | Injected | 584 | 617 | 670 | 412 | 462 | 472 |
| % change | Average | — | **+17** | — | — | **+25** | **+23** |
| | Maximum | -15 | +29 | +50 | +9 | +30 | +46 |
| | variations | +0·3 | +4 | -21 | -6 | +20 | +11 |
| *Heart*, mg. | Controls | 928 | 899 | 918 | 598 | 626 | 605 |
| | Injected | 862 | 868 | 930 | 602 | 656 | 653 |
| % change | Average | — | — | — | — | **+5** | **+8** |
| | Maximum | -14 | -8 | +15 | +10 | +6 | +19 |
| | variations | +0·2 | +1 | -15 | -8 | +4 | +1 |

Litters 11, 13, 16, and 19–25 were killed by bleeding from the abdominal aorta, the remaining litters being killed by coal gas. The difference between the actual weights of some organs in these two groups of litters was considerable (*e.g.* liver). However, since, in every case, the control rats were litter-mates of the injected groups, the percentage changes were comparable. Therefore, general averages of each group were made, irrespective of the method of killing, the results being summarised in Tables II–VIII. The tables are constructed on the same principle as previously [*e.g.* Korenchevsky *et al.*, 1932, Table II, p. 2099]. The average weights and the average percentage changes in weight in each group of each litter were obtained for each organ. Then general means were obtained by summing the average weights or average percentage changes and dividing by the number of litters. These general means are given in the tables and therefore the percentage changes are slightly different from the results calculated from the general means of the weights given in the tables in the two first lines for each organ. In addition to the average percentage change, the greatest variations in the percentage change, above and below the mean value, which were observed in the litters, are given in the 4th and 5th lines for each organ. Where the percentage changes in the weight of the organ showed an increase (+) in some, and a decrease (−) in other litters, no average is made, a blank, signifying non-constant changes, being used in place of the average figure (3rd line for each organ). For example, in Table II, last column, the average weight of the testes as compared with the average for the control litter-mates was 25 % larger in one litter and 24 % smaller in another. No average figure of the percentage change, therefore, could justifiably be given. Thus these tables, although economising space, give an accurate representation of the degree, variations and constancy of the changes obtained. Our long experience in applying our technique to the study of changes in the weights of organs has shown us that, when this technique is strictly followed in all details, even small changes may be significant and specific if they are constant.

EFFECT ON NORMAL RATS.

Each litter was divided into a control group and a group injected with 20, 60 or 180 international units of oestrin per day for a period varying from 29 to 43 days (Table I). Although the injections were started before the age of sexual maturity, the rats should have been mature by the last injection, since they were killed at 57–66 days old, by which age the rats of our stock are sexually mature.

*Influence on body weight, fat deposition and food intake.* As can be seen from Table I, the rats injected with daily doses of 60 or 180 I.U. weighed less and had a smaller gain in weight than their control litter-mates.

A rough estimate of food intake did not show any noticeable change in the appetite of rats injected with 20 I.U. The food intake (Table III) was therefore only recorded in the litters receiving 60 and 180 units of oestrin. The actual food intake was decreased on the average by 11 % in all the injected rats. When calculated per unit of body weight, however, the food intake was unchanged or, in most cases, slightly increased. The decreased appetite therefore could only have been partly responsible for the retarded growth and decreased gain in weight. Probably excessive amounts of oestrin injected into normal male rats injure in some way the catabolic processes in the organism. This injurious effect is probably related to the size of the dose, for the gain in body weight per 10 g. of actual food intake was as follows: in two groups of controls, 1·1 and 1·1 g., in the group injected with 60 units, 1·0 g. and in the group injected with 180 units, 0·9 g., *i.e.* the definite decrease of about 19 % was only found in the group receiving the large dose, although the decrease in actual food intake was practically identical in the two groups (Table III).

Fat deposition was not tabulated since the changes, which were mostly small, were not constant. Out of seven litters, there was a decrease in five of from 4 to 26 %, and increases in two of 20 and 70 % respectively (calculated per unit of body weight). Such a degree of fluctuation in fat deposition may occur physiologically, as has been shown in our previous paper [Korenchevsky and Dennison, 1934, pp. 240, 241].

*Influence on sexual organs.* In agreement with the previous workers mentioned above, we have found injections of large doses of oestrin to have a depressing effect on the actual weights of all the sexual organs (Table II). The comparatively small dose of 20 I.U. did not produce a constant or large effect. The depressing effect of doses of 60 or 180 I.U. was greatest in the case of seminal vesicles and prostate and least in the testes, the influence on the penis and preputial glands being intermediate. When calculated per unit of body weight the influence was still evident in the prostate and seminal vesicles, but the changes in the testes, penis and preputial glands became inconstant or small. It must be taken into consideration that a decreased food intake can produce similar changes in the weight of the sexual organs [Sampson and Korenchevsky, 1932], but not to such an extent as was observed in the present experiment. Therefore, if there was any injurious effect on the sexual organs of reduced appetite, the chief cause of the changes in these organs was the specific injurious effect of oestrin and not of the decreased food intake.

*Effects on adrenals and hypophysis.* As can be seen from Table III, in male rats the adrenals and hypophysis belong to those endocrine organs the weight of which is influenced in every case (see figures calculated per unit of body weight) by both the small and large doses used. The maximum variations are not so large in the adrenals as in the hypophysis, but for both organs the

hypertrophy was considerable in most of the injected rats. The changes in the actual weight are less pronounced in most groups and, in the case of the hypophysis, less constant.

From the foregoing, the changes in these two organs are seen to be the results of the specific influence of oestrin.

*Effects on thymus and spleen.* The figures in Tables II and III show that the changes obtained were comparatively small and were more definite and constant in the spleen than in the thymus. A slight or moderate increase in the weight of the spleen was observed in most of the rats injected with 20 and 60 I.U. of oestrin and in some cases though to a smaller degree, in the rats injected with the very large dose of 180 I.U. In the thymus a constant and more significant delay in involution (per unit of body weight) was noticeable only in the group injected with 60 I.U. of oestrin, while with 20 units this effect was very small and with 180 units was both small and variable. The chief reason for giving the data relating to these organs is that the male sexual hormone increases the speed of involution of the thymus in both normal and castrated rats, castration delaying this involution [Korenchevsky *et al.*, 1933; Korenchevsky and Dennison, 1934], while a very slight decrease in the weight of the spleen was found in most castrated rats [Korenchevsky and Dennison, 1934].

More numerous experiments, however, using different doses of oestrin, are necessary before the delay in involution of the thymus obtained with some doses can be considered to be specific.

*Effects on liver, kidneys and heart.* The effects were similar in these three organs (Table III). The actual weights of these organs were slightly decreased, while when calculated per unit of body weight these changes became less pronounced or were replaced by very small increases in weight. Changes of this type in the liver and kidneys are typical of rats in which the appetite is decreased (our unpublished experiments). It is therefore probable that these changes were chiefly due not to the specific effect of oestrin but to the decreased food intake. The possible significance of small changes in the heart will be discussed later.

### Effect on castrated rats.

In our previous papers we have studied the effect on castrated rats of testicular hormone injected for 7 days (chiefly for purposes of assay) and for the longer period of 3 weeks. Similarly, the injections of oestrin in the following experiments were made for a period of 7 days (Tables V and VII) and for longer periods of 28–43 days, after which period all the changes might be expected to be more pronounced (Tables VI and VIII). All the rats were castrated before sexual maturity and killed from 36 to 80 days after castration, at ages varying in different litters from 61 to 106 days (Table IV).

A study was made of the effects of four different doses 10, 20, 60 and 180 I.U. of oestrin.

*Effects on body weight, fat deposition and food intake.* The effect on the gain in body weight was noticeable in the long duration experiments in all the groups injected with 20, 60 and 180 units of oestrin. Thus, on the average, the gains in weight in the three groups mentioned above were 88, 83 and 86 g. respectively, while in the corresponding control litter-mates they were 127, 114 and 127 g. In the short duration experiments no definite effect was noticed in the groups injected with 10 or 20 units, but the check in the gain in body weight was significant in most of the rats injected with 60 and 180 units.

Changes in the food intake were recorded in only 3 litters, Nos. 11, 13 and 16, and since in these the food intake was decreased in 2 litters (injected with

20 units and 180 units of oestrin) and increased in the third (injected with 60 units of oestrin), more numerous experiments are required for definite conclusions.

Changes in the fat deposition were small. In the 7-day experiments the change in the amount of retroperitoneal fat did not exceed 36 %, 6 litters showing an increase and 3 a decrease. Of the 7 litters injected for a longer period the amount of retroperitoneal fat was slightly increased in 4 litters and decreased in 3 litters, the changes per unit of body weight in no litter exceeding the same figure of 36 %. Since such variations in the fat deposition are within the limits of physiological fluctuations, they are not given in the tables.

*Effect on sexual organs.* The effect on the sexual organs of castrated rats (Tables V and VI) was the reverse of that on normal rats, namely a constant increase in the weight of the prostate and seminal vesicles and a less constant increase in the weight of the penis and preputial glands. Between the doses of 10 and 60 units, small and large doses were followed by a corresponding difference in the effect. This difference in the response was small or not present in most organs between doses of 60–180 units. No direct proportional relation between the dose and the reaction could be seen however, except perhaps an approximate average percentage increase in the prostate or prostate with seminal vesicles in rats injected with 20 units as compared with rats injected with 60 units (Tables V and VI). The changes obtained were largest in the seminal vesicles and smallest in the penis.

The changes in the preputial glands were constant with large doses only in the 7-day experiments, becoming variable after prolonged injection, and are therefore not included in Table VI. For example, as compared with their control litter-mates the preputial glands (per unit of body weight) were increased by 13 and 30 % in 2 litters injected with 20 units of oestrin, increased by 56 % and decreased by 15 % in 2 litters injected with 60 units and decreased by 27 % and increased by 51 and 126 % in 3 litters injected with 180 units. Large variations, therefore, are obtained in preputial glands. This may partly be explained by the fact that these glands are difficult to dissect accurately, and the secretion is easily squeezed out during the dissection. The increase in the weight of the prostate was comparatively small, especially with small doses, being absent with 10 units and from 8 to 17 % with 20 units in 7-day experiments.

The effect on seminal vesicles of long duration injections was much greater than that of short duration injections, the former being, on the average, from two to five times the latter. The effect on the prostate of prolonged injection in most cases did not exceed, and was usually less than, double that obtained with 7 days of injection.

In the penis and preputial glands no definite or constant difference could be seen between the effects of short and long duration experiments.

*Effect on hypophysis.* As in normal rats, the weight of the hypophysis in castrated rats nearly always increased after the injection of oestrin both for 7 days and for the longer period, the increase however sometimes being small (Tables VI and VII). On the average the larger doses were followed by greater hypertrophy of the hypophysis only in the longer experiments, in which also the largest increases in this organ occurred (up to 72 %).

*Effect on thymus.* An increase in the involution of the thymus, although not constant or large, was present in most of the injected rats, especially after the injection of large doses for 7 days and after prolonged treatment. In castrated rats, therefore, the effect of oestrin on the thymus, though less constant, was similar to that of testicular hormone.

*Effects on the liver, kidneys, spleen and heart.* No definite changes were observed in the liver. In most rats, injections of oestrin slightly increased the weight of the kidneys and spleen, the changes in the spleen thus being similar to those obtained in normal rats.

If any significance can be ascribed to the very small increase in weight observed in the heart in most injected rats, this is also similar to that observed in normal rats.

*Note on the effects of oestrone on the weights of organs in castrated female rats.* In order to obtain some idea of the changes in the organs of castrated females as compared with those in males, a preliminary experiment was carried out with one litter of females, which was killed 50 days after castration at the age of 97 days. A total of 2340 I.U. of oestrin per rat was injected during a period of 23 days. In the injected rats, in addition to the usual large increase in the size of the uterus, an increase per unit of body weight as compared with the control litter-mates was obtained in the hypophysis (by 83 %) and in the kidneys (by 15 %). The changes were very small in all the other organs.

## Discussion.

It is clear, from the results obtained, that oestrin in the doses used had a quite definite effect on the gain in body weight, the weight of the sexual organs, of the adrenals and of the hypophysis of male rats. The hypertrophy of the hypophysis in both normal and castrated rats and of the adrenals in normal rats was an unexpected result of the injection of oestrin. We have previously shown that castration is always followed by such changes in these endocrine organs. The effect of oestrin on the hypophysis of normal or castrated rats may, therefore, be compared with that of castration, irrespective of the presence of the testes. The effect of oestrin on the adrenals of normal rats is also similar to that of castration. Without histological examination of the glands (which is now in progress) it is not possible to give a correct interpretation of these results.

In normal rats oestrin checks the development of the sexual organs. Moore and Price [1932] explain this by the depressing influence of oestrin on the secretion of the hormone of the anterior lobe of the hypophysis, since the simultaneous administration of the gonad-stimulating hormone, or making transplants of the hypophysis, is followed by the normal development of the sexual organs.

It is rather difficult to relate a depression in the activity of hypophysis with the hypertrophy of this gland which we obtained as the result of the injection of oestrin. However, the possibility of the depression of the special cells producing the gonad-stimulating hormone together with the hypertrophy of the other cells of the hypophysis cannot be excluded.

Of the sexual organs oestrin has the greatest effect on the seminal vesicles, which respond both to increase in the dose and in the period of injection. The response of the prostate to oestrin is much less, and the increase in reaction on increasing the dose or period of injection is also less than in the case of the seminal vesicles.

This sensitivity to oestrin, which is great in the case of seminal vesicles and only slight in the prostate, together with the occurrence of both male and female hormones in both sexes, suggests that, of these two glands, the prostate should be used for the assay of testicular hormone and the seminal vesicles as evidence of the possible presence of oestrin in the preparation (though both these organs are sensitive to testicular hormone).

Using the method of assay, previously proposed for male sexual hormone, but using the percentage increase in the weight of the prostate alone (Table V), doses of 60 and 180 I.U. of oestrin would contain, on the average, about 0·75 and 1 R.U. of male sexual hormone activity, while as judged by the percentage increase of the seminal vesicles they would contain about 2·75 R.U. We shall return to this problem in our next paper on the effect of the combined injection of oestrin and testicular hormones.

The stimulation of the penis and preputial glands by oestrin is not so regular as that of the seminal vesicles and prostate.

With regard to the changes in weight of the other organs no definite conclusion as to their specificity can be drawn until the histological examination is completed. Among the more constant changes were the slight increase in weight of the spleen and kidneys. The presence of a definite decrease in the weight of the heart of animals killed a long time after castration (oxen and geldings) necessitates an accurate investigation of this organ in all experiments dealing with the study of the sexual inner secretions. This question has been discussed in detail elsewhere [Korenchevsky and Dennison, 1934, p. 245]. In the present experiments, the increase in the weight of the heart in rats injected with oestrin was very small and not always constant.

## Summary.

1. The effect of subcutaneous injections of pure crystalline oestrone was studied in 39 normal and 73 castrated rats.

2. In *normal rats* large daily doses (60 and 180 I.U.) of oestrone decreased the appetite and gain in body weight without any definite change in the fat deposition, depressed development of the sexual organs and produced hypertrophy of the adrenals and hypophysis. Small and less definite changes were found in the thymus and spleen (an increase with some doses), liver and kidney (decrease in actual weight). The changes in these last two organs were explained by a decreased food intake.

3. In *castrated rats*, in both 7-day and long period experiments, oestrone decreased the gain in body weight of most animals (except with 10 and 20 I.U. in 7-day experiments), but did not produce definite changes in the fat deposition, greatly increased the weight of the seminal vesicles, slightly increased that of the prostate and, less constantly, that of the penis and preputial glands and produced hypertrophy of the hypophysis. With larger doses, or after a prolonged period of injection, the rate of involution of the thymus was increased in most rats. Less definite changes (slight increase with some doses) were observed in the kidneys and spleen. The changes in the remaining organs investigated were very small.

4. It may therefore be concluded that the presence of oestrin in the male organism has a definite effect on some of the organs which are also influenced by testicular hormone.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due.

REFERENCES.

Burrows and Kennaway (1934). *Amer. J. Cancer*, **20**, 48.
Dingemanse (1931). *Proc. II Inter. Congress Sex. Res.* 374.
Freud (1933). *Biochem. J.* **27**, 1438.
Korenchevsky and Dennison (1934). *J. Path. Bact.* **38**, 231.
—— —— and Kohn-Speyer (1932). *Biochem. J.* **26**, 2097.
—— —— —— (1933). *Biochem. J.* **27**, 557.
Lacassagne (1933). *Compt. Rend. Soc. Biol.* **113**, 590.
Loewe, Randenbusch, Voss and Lange (1932). *Biochem. Z.* **250**, 50.
Moore and Price (1932). *Amer. J. Anat.* **50**, 13.
Sampson and Korenchevsky (1932). *Biochem. J.* **26**, 1322.
Womack and Koch (1932). *Endocrinology*, **16**, 273.
Zondek (1934). *Nature*, **133**, 209.
—— and Euler (1934). *Skand. Arch. Physiol.* **67**, 259.

# CXCVII. THE EFFECT ON MALE RATS OF THE SIMULTANEOUS ADMINISTRATION OF MALE AND FEMALE SEXUAL HORMONES AND THE RELATION TO THE ASSAY OF THE HORMONES.

By VLADIMIR KORENCHEVSKY and MARJORIE DENNISON.

*From the Lister Institute, London.*

(*Received July 1st, 1934.*)

The experiments on rats, of Moore and Price [1932] and of Freud [1933], relating to this question have already been referred to by us [1934, 2].

Schoeller and Gehrke [1933] also performed some interesting experiments on birds. In hens, injected with 10 C.U. of testicular hormone daily for 4 weeks, the comb-growth increased 23 %, while in those injected with 4000 M.U. of oestrin per week for 6 weeks the size of the comb decreased by 11 %. When capons were injected for 2 days, with testicular hormone (2 C.U.) and oestrin (5000 M.U.) simultaneously, the comb-growth on the third day was 50 % greater than that obtained with the same dose of testicular hormone alone. Thus, although in capons the degree of the reaction was less and the dose of oestrin used much larger, the simultaneous injection of oestrin with testicular hormone was found to have a similar stimulating effect on the comb-growth of capons as has been observed by Freud on the seminal vesicles of castrated rats. Freud performed his experiments on 3–5 weeks' old rats 3 days after castration, when atrophy of the seminal vesicles was not complete, used litter-mates only "as far as possible" and weighed the seminal vesicles fresh. This is perhaps the partial explanation of some of the "paradoxical results" mentioned on p. 1442 and seen in some of the other tables in Freud's very interesting paper.

In the present paper, besides a repetition of Freud's experiments on the seminal vesicles of castrated rats, we have studied the influence in castrated male rats of combined injections of testicular hormone and oestrin on the weight of the other secondary sexual organs, body weight, fat deposition and the endocrine and some of the other organs, which are influenced to a greater or less extent by testicular hormone as we have previously shown [Korenchevsky *et al.*, 1933, 1].

## Technique.

Experiments were performed on 73 castrated rats belonging to 16 litters. The number of rats in the litter, the final age, the age at castration and the number of days after castration that the animals were killed, the final weights and the preparation of testicular hormone injected are given in Table I.

A study of the effect of daily injections during 7 days was made in order to decide what changes in the effects of testicular hormone will be caused by the presence of oestrin in a preparation under assay.

A longer period of injection of 21 days was also used in order that more pronounced effects might be obtained for the study of the effects of the injection.

As in the previous experiments, crystalline oestrone[1] dissolved in olive oil was used for the injections. Two preparations of testicular hormone were used: one, designated in the tables as "Pr," was supplied by Messrs Schering, Ltd.; the other, designated as "H," was sent from Holland by Messrs Organon, Ltd.[2] The latter preparation is controlled in the pharmaco-therapeutic laboratory of Amsterdam University. Both preparations were used in doses of 1·5 C.U., though this does not imply that their actual strength in C.U. was the same, since the method of assay on capons is different in these two laboratories. In Tables II, IV, V, VI and VII, the average results obtained in the individual litters are given, while Table III gives only the general means averaged from the litters 6–13 and is constructed in the same way as the tables in the previous paper.

Table I. *Age, average weight and period of injections of castrated rats.*

| No. of litter | No. of rats in litter | Period of injections Days | Age in days: At castration | Age in days: When killed | No. of days after castration when killed | Final weights, g.: Controls | Test. hor. alone | Test. hor. + oes. 20 | Test. hor. + oes. 60 | Test. hor. + oes. 180 | Testicular hormone preparation used |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 7 | 7 | 25 | 83 | 58 | — | 301 | — | 291 | 294 | |
| 2 | 6 | 7 | 25 | 57 | 32 | — | 208 | — | 193 | 182 | |
| 3 | 4 | 7 | 22 | 54 | 32 | — | 188 | — | 176 | — | "Pr" |
| 4 | 7 | 21 | 21 | 83 | 62 | 296 | 330 | — | — | 273 | |
| 5 | 5 | 21 | 21 | 83 | 62 | 241 | 305 | — | — | 255 | |
| 6 | 3 | 7 | 30 | 86 | 56 | — | 306 | 286 | — | — | |
| 7 | 5 | 7 | 23 | 76 | 53 | — | 263 | 272 | — | — | |
| 8 | 4 | 7 | 23 | 78 | 55 | — | 304 | — | 275 | — | |
| 9 | 4 | 7 | 23 | 79 | 56 | — | 249 | — | 274 | — | |
| 10 | 4 | 7 | 25 | 73 | 48 | — | 282 | — | 265 | — | |
| 11 | 4 | 7 | 23 | 77 | 54 | — | 269 | — | — | 274 | "H" |
| 12 | 3 | 7 | 24 | 77 | 53 | — | 259 | — | — | 241 | |
| 13 | 5 | 7 | 30 | 74 | 44 | — | 237 | — | 240 | 222 | |
| 14 | 5 | 21 | 24 | 95 | 71 | 345 | 357 | — | 335 | — | |
| 15 | 3 | 21 | 24 | 92 | 68 | — | 308 | — | 294 | — | |
| 16 | 4 | 21 | 21 | 89 | 68 | — | 327 | — | — | 305 | |

SEVEN-DAY EXPERIMENTS.

The controls in these experiments were injected with testicular hormone alone; the litter-mates received testicular hormone *plus* varying doses of oestrin.

*Influence on gain in body weight and fat deposition.* These data are not included in the table, but it is possible to say that on the average, as compared with the litter-mates receiving testicular hormone alone, the addition of the large dose of oestrin of 180 I.U. was followed by a decreased gain in body weight and slightly decreased fat deposition. For example, three control groups of rats injected with "H" alone in 7 days gained in weight 27, 19 and 22 g. respectively, while their litter-mates injected with "H"+180 I.U. of oestrin gained 11, 5 and 3 g. respectively. In the same litters, the corresponding weights of retro-peritoneal fat were, in controls injected with "H" alone, 8·6, 9·2 and 9·0 g. and, in the litter-mates injected with "H"+180 I.U. of oestrin, 6·1, 8·2 and 6·1 g.

[1] Referred to as oestrin throughout the body of the paper.

[2] We wish to express our thanks to the Department of Biological Standards for oestrin and to Messrs Organon, Ltd., and Schering, Ltd., for testicular hormone preparations.

Table II. *Effect on sexual organs and hypophysis of 7 days of injections of testicular hormone preparation "Pr" alone or in combination with oestrone.*

| No. of litter | Actual: Weights, mg. | | | Actual: % change | | Per 200 g. body weight: Weights, mg. | | | Per 200 g. body weight: % change | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pr 1·5 | Pr 1·5 Oes. 60 | Pr 1·5 Oes. 180 | Pr 1·5 Oes. 60 | Pr 1·5 Oes. 180 | Pr 1·5 | Pr 1·5 Oes. 60 | Pr 1·5 Oes. 180 | Pr 1·5 Oes. 60 | Pr 1·5 Oes. 180 |
| *Prostate with seminal vesicles.* | | | | | | | | | | |
| 1 | 147 | 149 | 167 | + 1 | +14 | 98 | 102 | 113 | + 4 | +15 |
| 2 | 131 | 148 | 154 | +13 | +18 | 126 | 151 | 169 | +20 | +34 |
| 3 | 144 | 165 | — | +15 | — | 154 | 189 | — | +23 | — |
| Av. | **141** | **154** | **161** | **+10** | **+16** | **126** | **147** | **141** | **+16** | **+25** |
| *Prostate.* | | | | | | | | | | |
| 1 | 117 | 119 | 134 | + 2 | +15 | 78 | 82 | 91 | + 5 | +17 |
| 2 | 105 | 115 | 116 | +10 | +11 | 101 | 119 | 128 | +18 | +27 |
| 3 | 120 | 126 | — | + 5 | — | 128 | 144 | — | +13 | — |
| Av. | **114** | **120** | **125** | **+ 6** | **+13** | **102** | **115** | **110** | **+12** | **+22** |
| *Seminal vesicles.* | | | | | | | | | | |
| 1 | 30 | 30 | 32 | ± 0 | + 7 | 20 | 21 | 22 | + 5 | +10 |
| 2 | 26 | 31 | 38 | +19 | +46 | 25 | 32 | 42 | +28 | +68 |
| 3 | 24 | 39 | — | +63 | — | 26 | 45 | — | +73 | — |
| Av. | **27** | **33** | **35** | **+27** | **+27** | **24** | **33** | **32** | **+35** | **+39** |
| *Penis.* | | | | | | | | | | |
| 1 | 172 | 160 | 182 | − 7 | + 6 | 115 | 110 | 124 | − 4 | + 8 |
| 2 | 128 | 138 | 133 | + 8 | + 4 | 123 | 143 | 147 | +16 | +20 |
| 3 | 125 | 117 | — | − 6 | — | 133 | 134 | — | + 1 | — |
| Av. | **142** | **138** | **158** | — | **+ 5** | **124** | **129** | **136** | — | **+14** |
| *Preputial glands.* | | | | | | | | | | |
| 1 | 87 | 110 | 107 | +26 | +23 | 58 | 76 | 73 | +31 | +26 |
| 2 | 102 | 93 | 114 | − 9 | +12 | 98 | 96 | 125 | − 2 | +28 |
| 3 | 106 | 107 | — | + 1 | — | 112 | 122 | — | + 9 | — |
| Av. | **98** | **103** | **111** | — | **+18** | **89** | **98** | **66** | — | **+27** |
| *Hypophysis* | | | | | | | | | | |
| 1 | 16·5 | 17·7 | 18·5 | + 7 | +12 | 10·9 | 12·2 | 12·5 | +12 | +15 |
| 2 | 11·9 | 12·6 | 13·0 | + 6 | + 9 | 11·5 | 12·3 | 14·3 | + 7 | +24 |
| 3 | 9·6 | 12·3 | — | +28 | — | 10·7 | 14·0 | — | +31 | — |
| Av. | **12·7** | **14·2** | **15·8** | **+10** | **+11** | **11·0** | **12·8** | **13·4** | **+17** | **+20** |

*The influence on the secondary sexual organs.* As can be seen from Tables II and III, the effect on the weight of the prostate of the addition of oestrin to testicular hormone "Pr" (Table II) or "H" (Table III), was small and similar in degree. The largest increase in weight of the prostate after the addition of oestrin to testicular hormone "Pr" was 27 % in litter 2 (Table II). With preparation "H," in several cases there was no effect at all on the prostate and the maximum increase was 20 % (Table III). The effect of increasing the dose of oestrin, when any, was small.

On the other hand, in the case of the seminal vesicles the addition of 60 or 180 I.U. of oestrin to the testicular hormone always greatly increased the effect of preparation "H," and in most cases and to a smaller degree, that of preparation "Pr." In the case of "H" the addition of larger doses of oestrin produced a larger effect on the seminal vesicles but not on the prostate. Thus, difference of action on seminal vesicles indicates a difference in the preparations "Pr" and "H." The effect of the addition of oestrin on changes in

Table III. *Effect on sexual organs and hypophysis of 7 days of injection of testicular hormone preparation "H" in combination with oestrone.*

| | | Weights | | | | | |
|---|---|---|---|---|---|---|---|
| | | Actual | | | Per 200 g. body weight | | |
| | | 20 u. | 60 u. | 180 u. | 20 u. | 60 u. | 180 u. |
| *Prostate with sem. ves.*, mg. | Controls | 122 | 116 | 120 | 85 | 87 | 94 |
| | Injected | 122 | 134 | 140 | 88 | 104 | 115 |
| % change | Average | — | +16 | +17 | — | +20 | +22 |
| | Maximum variations | +9 | +19 | +23 | +6 | +33 | +30 |
| | | −8 | +13 | + 7 | −1 | + 9 | +17 |
| *Prostate*, mg. | Controls | 101 | 98 | 105 | 71 | 73 | 82 |
| | Injected | 95 | 105 | 106 | 69 | 80 | 87 |
| % change | Average | −5 | + 8 | — | −3 | + 9 | — |
| | Maximum variations | −9 | +11 | +9 | −3 | +20 | +17 |
| | | −1 | + 3 | −2 | −3 | + 1 | − 2 |
| *Sem. ves.*, mg. | Controls | 22 | 18 | 16 | 16 | 14 | 13 |
| | Injected | 27 | 29 | 34 | 19 | 23 | 28 |
| % change | Average | — | + 64 | +110 | +24 | +73 | +115 |
| | Maximum variations | +50 | +113 | +157 | +47 | +140 | +136 |
| | | − 4 | + 36 | + 67 | ± 0 | + 47 | + 79 |
| *Penis*, mg. | Controls | 142 | 133 | 131 | 100 | 100 | 103 |
| | Injected | 128 | 145 | 134 | 92 | 112 | 110 |
| % change | Average | −10 | + 9 | — | − 8 | +12 | + 7 |
| | Maximum variations | −11 | +18 | +8 | −10 | +18 | +12 |
| | | − 8 | + 5 | −6 | − 5 | + 5 | + 2 |
| *Hypophysis*, mg. | Controls | 12·3 | 12·0 | 11·6 | 8·8 | 9·0 | 9·1 |
| | Injected | 14·7 | 13·9 | 14·2 | 10·6 | 10·6 | 11·7 |
| % change | Average | +20 | +16 | +23 | +24 | +18 | +29 |
| | Maximum variations | +30 | +39 | +30 | +40 | +26 | +39 |
| | | +10 | + 1 | +10 | + 7 | +19 | +19 |

*Note.* In this table "controls" denotes rats receiving testicular hormone only; "injected" receiving the same dose of testicular hormone + various doses of oestrin, as shown in the heading of the table.

the penis and preputial glands was less constant but similar in degree to that on prostate alone.

*Influence on hypophysis.* One of the effects of the injection of oestrin into male normal and castrated rats was an increase in the weight of the hypophysis, as has been shown in the previous paper. This effect was again evident when oestrin was added to testicular hormone preparation, although the percentage increase in weight was sometimes small (Tables II and III).

*Effect on other organs.* Although the other organs were also weighed, no definite or constant changes in the weights were found to occur after the addition of oestrin to testicular hormone preparations, as compared with the litter-mates injected with testicular hormone alone.

### Twenty-one day experiments.

These experiments were performed on five litters, Nos. 4 and 5 containing 12 rats treated with testicular preparation "Pr" (Tables I, IV and VI) and Nos. 14, 15 and 16, also containing 12 rats, injected with testicular preparation "H" (Tables I, V and VII).

All the experiments were performed at the same time, using the same solution of oestrin, a point of importance when considering some of the differences between the results obtained with preparation "Pr" and preparation "H."

Table IV. *Effect on sexual organs and hypophysis of 21 days of injections of testicular hormone preparation "Pr" alone or in combination with oestrone.*

| No. of litter | Actual: Weights, mg. Control | Actual: Weights, mg. Pr 1·5 | Actual: Weights, mg. Pr 1·5 Oes. 180 | Actual: % change on controls Pr 1·5 | Actual: % change on controls Pr 1·5 Oes. 180 | Per 200 g. body weight: Weights, mg. Control | Per 200 g. body weight: Weights, mg. Pr 1·5 | Per 200 g. body weight: Weights, mg. Pr 1·5 Oes. 180 | Per 200 g. body weight: % change on controls Pr. 1·5 | Per 200 g. body weight: % change on controls Oes. 180 | Per 200 g. body weight: % change on group Pr 1·5 Oes. 180 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Prostate with seminal vesicles.* | | | | | | | | | | | |
| 4 | 66 | 201 | 222 | +205 | +236 | 45 | 122 | 163 | +171 | +262 | +34 |
| 5 | 61 | 191 | 263 | +213 | +331 | 51 | 126 | 206 | +147 | +304 | +64 |
| Av. | **64** | **196** | **243** | **+209** | **+284** | **48** | **124** | **185** | **+159** | **+283** | **+49** |
| *Prostate only.* | | | | | | | | | | | |
| 4 | 55 | 163 | 168 | +196 | +206 | 37 | 100 | 123 | +170 | +232 | +23 |
| 5 | 51 | 157 | 208 | +208 | +308 | 42 | 104 | 163 | +148 | +288 | +57 |
| Av. | **53** | **160** | **188** | **+202** | **+257** | **40** | **102** | **143** | **+159** | **+260** | **+40** |
| *Seminal vesicles only.* | | | | | | | | | | | |
| 4 | 11 | 38 | 54 | +246 | +391 | 8 | 24 | 40 | +200 | +400 | +67 |
| 5 | 10 | 34 | 55 | +240 | +450 | 8 | 22 | 43 | +175 | +434 | +96 |
| Av. | **11** | **36** | **55** | **+243** | **+421** | **8** | **23** | **42** | **+188** | **+417** | **+82** |
| *Penis.* | | | | | | | | | | | |
| 4 | 85 | 143 | 145 | + 68 | + 71 | 57 | 92 | 115 | + 61 | + 88 | +16 |
| 5 | 95 | 131 | 130 | + 38 | + 37 | 79 | 87 | 103 | + 10 | + 30 | +18 |
| Av. | **90** | **137** | **138** | **+ 53** | **+ 54** | **68** | **90** | **109** | **+ 36** | **+ 59** | **+17** |
| *Preputial glands.* | | | | | | | | | | | |
| 4 | 52 | 125 | 127 | +140 | +144 | 35 | 76 | 93 | +117 | +166 | +22 |
| 5 | 44 | 163 | 166 | +271 | +277 | 36 | 107 | 129 | +197 | +258 | +21 |
| Av. | **48** | **144** | **147** | **+206** | **+211** | **36** | **92** | **111** | **+157** | **+212** | **+22** |
| *Hypophysis.* | | | | | | | | | | | |
| 4 | 11·6 | 11·2 | 13·0 | − 3 | + 12 | 8·0 | 6·8 | 9·5 | − 15 | + 19 | +40 |
| 5 | 13·8 | 13·9 | 14·8 | + 1 | + 7 | 11·4 | 9·2 | 11·8 | − 19 | + 4 | +28 |
| Av. | **12·7** | **12·6** | **13·9** | — | **+ 10** | **9·7** | **8·0** | **10·6** | **− 17** | **+ 12** | **+34** |

Unfortunately the uninjected control rats of litters 15 and 16 had severe pneumonia and therefore, in accordance with our technique, these rats were excluded. Since there remained only the one control rat of litter 14, uninjected control rats of this experiment were not included in the tables. In Tables V and VII, therefore, the rats injected with testicular hormone alone (in the tables designated as "H 1·5") are used as the controls to those injected simultaneously with testicular hormone and oestrin. For the direct comparison of the results obtained with preparations "H" and "Pr," in the "Pr" experiments (Tables IV and VI) percentage changes per 200 g. of body weight were referred both to the uninjected control rats and to the rats injected with "Pr" alone. Therefore, the figures of percentage change per 200 g. of body weight in Tables V and VII can only be compared with those of the last column of Tables IV and VI.

*Influence on food intake, body weight and fat deposition.* It can be seen from Table VI that the actual food intake increased (up to 22 %) when "Pr" alone was injected and that this influence disappeared, or decreased, after the additional injection of oestrin. This latter effect is also shown in Table VII, although when calculated per unit of body weight the change in appetite is very small in both "Pr" and "H" experiments.

Table V. *Effects on sexual glands and adrenals of 21 days of injections of testicular hormone preparation "H" alone or in combination with oestrone.*

| No. of litter | Actual: Weights, mg. H 1·5 | Actual: Weights, mg. H 1·5 Oes. 60 | Actual: Weights, mg. H 1·5 Oes. 180 | Actual: % change H 1·5 Oes. 60 | Actual: % change H 1·5 Oes. 180 | Per 200 g. body weight: Weights, mg. H 1·5 | Per 200 g. body weight: Weights, mg. H 1·5 Oes. 60 | Per 200 g. body weight: Weights, mg. H 1·5 Oes. 180 | Per 200 g. body weight: % change H 1·5 Oes. 60 | Per 200 g. body weight: % change H 1·5 Oes. 180 |
|---|---|---|---|---|---|---|---|---|---|---|
| *Prostate with seminal vesicles.* | | | | | | | | | | |
| 14 | 153 | 160 | — | + 5 | — | 86 | 96 | — | +12 | — |
| 15 | 173 | 170 | — | - 2 | — | 112 | 116 | — | + 4 | — |
| 16 | 182 | — | 210 | — | +15 | 111 | — | 138 | — | +24 |
| Av. | **169** | **165** | **210** | — | **+15** | **103** | **106** | **138** | + 8 | **+24** |
| *Prostate only.* | | | | | | | | | | |
| 14 | 122 | 116 | — | - 5 | — | 69 | 70 | — | + 1 | — |
| 15 | 142 | 133 | — | - 6 | — | 92 | 91 | — | - 1 | — |
| 16 | 156 | — | 170 | — | + 9 | 96 | — | 111 | — | +16 |
| Av. | **140** | **125** | **170** | **- 6** | **+ 9** | **86** | **81** | **111** | — | **+16** |
| *Seminal vesicles only.* | | | | | | | | | | |
| 14 | 31 | 44 | — | +42 | — | 18 | 26 | — | +44 | — |
| 15 | 31 | 37 | — | +19 | — | 20 | 25 | — | +25 | — |
| 16 | 26 | — | 40 | — | +54 | 16 | — | 26 | — | +63 |
| Av. | **29** | **41** | **40** | **+31** | **+54** | **18** | **26** | **26** | **+35** | **+63** |
| *Penis.* | | | | | | | | | | |
| 14 | 142 | 125 | — | -12 | — | 80 | 74 | — | - 8 | — |
| 15 | 144 | 151 | — | + 5 | — | 94 | 103 | — | +10 | — |
| 16 | 153 | — | 138 | — | -10 | 94 | — | 91 | — | - 3 |
| Av. | **146** | **138** | **138** | — | **-10** | **89** | **89** | **91** | — | **- 3** |
| *Preputial glands.* | | | | | | | | | | |
| 14 | 72 | 80 | — | +11 | — | 40 | 48 | — | +20 | — |
| 15 | 93 | 111 | — | +19 | — | 60 | 75 | — | +25 | — |
| 16 | 100 | — | 108 | — | + 8 | 63 | — | 71 | — | +13 |
| Av. | **88** | **96** | **108** | **+15** | **+ 8** | **54** | **62** | **71** | **+23** | **+13** |
| *Adrenals.* | | | | | | | | | | |
| 14 | 81·1 | 88·0 | — | + 9 | — | 45·5 | 52·5 | — | +15 | — |
| 15 | 90·0 | 100·5 | — | +11 | — | 58·5 | 68·5 | — | +17 | — |
| 16 | 64·6 | — | 82·2 | — | +27 | 39·6 | — | 54·1 | — | +37 |
| Av. | **78·6** | **94·3** | **82·2** | **+10** | **+27** | **47·9** | **60·5** | **54·1** | **+16** | **+37** |

During the injection of "Pr" testicular hormone alone, as compared with the uninjected controls the total gain in body weight increased, but decreased after the addition of oestrin to both "Pr" and "H" preparations, as is shown by the following figures:

| | | | | | | |
|---|---|---|---|---|---|---|
| | Litter Nos. ... | 4 | 5 | 14 | 15 | 16 |
| Gain in body weight, g. | Controls | 75 | 56 | — | — | — |
| | "Pr" or "H" | 85 | 77 | 64 | 44 | 65 |
| | "Pr" or "H" + oestrin | 35 | 34 | 40 | 38 | 32 |

Corresponding changes were noted in the fat deposition. The weight of the retroperitoneal fat was increased with "Pr" alone (Table VI), but decreased with the additional injection of oestrin (Tables VI and VII).

Catabolic processes also seem to be favourably influenced by injections of testicular hormone alone and slightly depressed with additional injections of

Table VI. *Effect on thymus, liver, kidneys, heart, retroperitoneal fat and food intake of 21 days of injections of testicular hormone preparation "Pr" alone or in combination with oestrone.*

| No. of litter | Actual: Weights: Control | Actual: Weights: Pr 1·5 | Actual: Weights: Pr 1·5 Oes. 180 | Actual: % change on controls: Pr 1·5 | Actual: % change on controls: Pr 1·5 Oes. 180 | Per 200 g. body weight: Weights: Control | Per 200 g. body weight: Weights: Pr 1·5 | Per 200 g. body weight: Weights: Pr 1·5 Oes. 180 | Per 200 g. body weight: % change on controls: Pr 1·5 | Per 200 g. body weight: % change on controls: Pr 1·5 Oes. 180 | Per 200 g. body weight: % change on group: Pr 1·5 Oes. 180 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Thymus*, mg. | | | | | | | | | | | |
| 4 | 696 | 560 | 444 | −20 | −36 | 471 | 341 | 326 | −28 | −31 | − 4 |
| 5 | 445 | 634 | 346 | +43 | −22 | 369 | 416 | 270 | +13 | −27 | −35 |
| Av. | **571** | **597** | **395** | — | **−29** | **420** | **379** | **298** | — | **−29** | **−20** |
| *Liver*, g. | | | | | | | | | | | |
| 4 | 10·28 | 13·69 | 11·33 | +33 | +10 | 6·94 | 8·31 | 8·31 | +20 | +20 | ± 0 |
| 5 | 8·13 | 12·14 | 10·33 | +49 | +27 | 6·74 | 7·96 | 8·08 | +18 | +20 | + 2 |
| Av. | **9·21** | **12·92** | **10·83** | **+41** | **+19** | **6·84** | **8·14** | **8·20** | +19 | +20 | + 1 |
| *Kidneys*, g. | | | | | | | | | | | |
| 4 | 1·677 | 2·052 | 2·028 | +22 | +21 | 1·132 | 1·245 | 1·484 | +10 | +31 | +19 |
| 5 | 1·383 | 2·008 | 1·817 | +45 | +31 | 1·146 | 1·314 | 1·430 | +15 | +25 | + 9 |
| Av. | **1·530** | **2·030** | **1·923** | **+34** | **+26** | **1·139** | **1·280** | **1·457** | **+13** | **+28** | **+14** |
| *Heart*, mg. | | | | | | | | | | | |
| 4 | 813 | 934 | 803 | +15 | − 1 | 550 | 567 | 588 | + 3 | + 7 | + 4 |
| 5 | 663 | 859 | 826 | +30 | +25 | 549 | 563 | 651 | + 3 | +19 | +16 |
| Av. | **740** | **897** | **815** | **+23** | — | **550** | **565** | **620** | + 3 | **+13** | **+10** |
| *Retroperitoneal fat*, g. | | | | | | | | | | | |
| 4 | 12·34 | 15·59 | 11·35 | +26 | − 8 | 8·34 | 9·47 | 8·27 | +14 | − 1 | −13 |
| 5 | 8·63 | 13·50 | 10·06 | +56 | +17 | 7·15 | 8·88 | 7·71 | +24 | + 8 | −13 |
| Av. | **10·49** | **14·55** | **10·71** | **+41** | — | **7·75** | **9·18** | **7·99** | **+19** | — | **−13** |
| *Food intake*, g. | | | | | | | | | | | |
| 4 | 40·0 | 45·0 | 35·3 | +13 | −12 | 27·6 | 27·9 | 26·3 | + 1 | − 5 | − 6 |
| 5 | 34·1 | 44·7 | 34·9 | +31 | + 2 | 28·6 | 30·3 | 27·8 | + 6 | − 3 | − 8 |
| Av. | **37·1** | **44·9** | **35·1** | **+22** | — | **28·1** | **29·1** | **27·1** | **+ 4** | **− 4** | **− 7** |

oestrin, as is suggested by the following figures (g.) of gain in body weight per unit (10 g.) of food intake:

| Litter Nos. ... | 4 | 5 | 14 | 15 | 16 |
|---|---|---|---|---|---|
| Controls | 0·48 | 0·29 | — | — | — |
| "Pr" or "H" | 0·48 | 0·64 | 0·40 | 0·38 | 0·56 |
| "Pr" or "H" + oestrin | 0·29 | 0·28 | 0·30 | 0·30 | 0·43 |

Although in this experiment only litter 5 shows an increase on the injection of testicular hormone, litter 4 showing no change, in our earlier experiments an increase in gain in body weight per unit of food intake was found in all the litters injected with testicular hormone [Korenchevsky *et al.*, 1933, 1, p. 561, Table III]. It would seem therefore that, in general, testicular hormone had a slightly stimulating and oestrin a slightly depressing effect on the appetite, gain in body weight and fat deposition.

*Influence on the secondary sexual organs.* It can be seen from Table V that the addition of 180 I.U. of oestrin to the daily dose of "H" slightly increased the weight of the prostate, by 16 %, while when "Pr" was used the increases were 23 and 57 % in two litters respectively, on the average 40 % (Table IV). Thus, after prolonged injections, in contrast to the results of 7-day experiments,

Table VII. *Effects on hypophysis, thymus, kidneys, spleen, retroperitoneal fat and daily food intake of 21 days of injections of testicular hormone preparation "H" alone or in combination with oestrone.*

| | | Actual | | | | Per 200 g. body weight | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Weights | | | % change | | Weights | | % change | |
| No. of litter | H 1·5 | H 1·5 Oes. 60 | H 1·5 Oes. 180 | H 1·5 Oes. 60 | H 1·5 Oes. 180 | H 1·5 | H 1·5 Oes. 60 | H 1·5 Oes. 180 | H 1·5 Oes. 60 | H 1·5 Oes. 180 |
| *Hypophysis*, mg. | | | | | | | | | | |
| 14 | 14·6 | 15·6 | — | + 7 | — | 8·2 | 9·3 | — | +13 | — |
| 15 | 12·6 | 17·2 | — | +37 | — | 8·2 | 11·8 | — | +44 | — |
| 16 | 12·3 | — | 17·6 | — | +43 | 7·6 | — | 11·6 | — | +53 |
| Av. | **13·2** | **16·4** | **17·6** | **+22** | **+43** | **8·0** | **10·6** | **11·6** | **+29** | **+53** |
| *Thymus*, mg. | | | | | | | | | | |
| 14 | 586 | 562 | — | − 4 | — | 329 | 343 | — | + 4 | — |
| 15 | 721 | 481 | — | −33 | — | 468 | 329 | — | −30 | — |
| 16 | 650 | — | 425 | — | −35 | 396 | — | 279 | — | −30 |
| Av. | **652** | **522** | **425** | **−19** | **−35** | **394** | **336** | **279** | — | **−30** |
| *Kidney*, g. | | | | | | | | | | |
| 14 | 1·986 | 2·153 | — | + 8 | — | 1·112 | 1·285 | — | +16 | — |
| 15 | 2·037 | 2·221 | — | + 9 | — | 1·324 | 1·512 | — | +14 | — |
| 16 | 2·125 | — | 2·244 | — | + 6 | 1·297 | — | 1·473 | — | +14 |
| Av. | **2·049** | **2·187** | **2·244** | **+ 9** | **+ 6** | **1·244** | **1·399** | **1·473** | **+15** | **+14** |
| *Spleen*, mg. | | | | | | | | | | |
| 14 | 649 | 665 | — | + 3 | — | 364 | 396 | — | + 9 | — |
| 15 | 634 | 627 | — | − 1 | — | 412 | 429 | — | + 4 | — |
| 16 | 500 | — | 524 | — | + 5 | 306 | — | 342 | — | +12 |
| Av. | **594** | **646** | **524** | — | + 5 | **361** | **413** | **342** | **+ 7** | **+12** |
| *Retroperitoneal fat*, g. | | | | | | | | | | |
| 14 | 19·51 | 17·68 | — | − 9 | — | 10·94 | 10·54 | — | − 4 | — |
| 15 | 13·21 | 9·63 | — | −27 | — | 8·59 | 6·48 | — | −25 | — |
| 16 | 14·54 | — | 11·80 | — | −19 | 8·88 | — | 7·73 | — | −13 |
| Av. | **15·75** | **13·66** | **11·80** | **−18** | **−19** | **9·47** | **8·51** | **7·73** | **−15** | **−13** |
| *Daily food intake*, g. | | | | | | | | | | |
| 14 | 45·8 | 41·3 | — | −10 | — | 26·2 | 25·0 | — | − 5 | — |
| 15 | 36·6 | 36·8 | — | + 1 | — | 24·2 | 25·5 | — | + 5 | — |
| 16 | 43·9 | — | 42·0 | — | − 4 | 27·7 | — | 28·2 | — | + 2 |
| Av. | **42·1** | **39·1** | **42·0** | — | **− 4** | **26·1** | **25·3** | **28·2** | — | **+ 2** |

the effect on the prostate of some testicular preparations may be considerably increased by the addition of large doses of oestrin. The addition of 60 I.U. of oestrin to the testicular preparation "H" did not give any change in the weight of the prostate and we made no experiments with this dose of oestrin in combination with "Pr."

The effect on the seminal vesicles of the addition of oestrin was similar to that in the 7-day experiments; in short a considerable increase in the weight of this organ follows the addition of oestrin to the injection of testicular hormone. A larger dose produced a greater reaction (Table V).

The changes in the prostate with seminal vesicles were closer to those in the prostate alone than to those in the seminal vesicles alone (owing mainly to the relative weights of these two organs).

The influence of the additional injection of oestrin on the weight of the penis and preputial glands was small, being practically absent in the penis of rats injected with "H"+oestrin.

*Effect on hypophysis.* After the addition of oestrin this endocrine organ increased in every litter (Table IV and VII), *i.e.* this typical effect of oestrin was again evident and the simultaneous administration of testicular hormone did not obscure it.

*Effect on thymus.* The addition of oestrin to "Pr" and "H" accelerated the involution of the thymus in most rats, a result which is in good agreement with the results obtained in the previous paper, on the injection of oestrin into castrated rats.

*Effects on the kidney, spleen and heart.* The effects on these organs of the addition of oestrin to testicular hormone were very small, but when present (Tables VI and VII) were similar to those described in the previous paper on the injection of oestrin into castrated rats.

The possible specificity of this effect of oestrin on the kidneys is supported by our earlier experiments, in which it was found that "Pr" injected for 21 days produced an increase in the weight of the kidneys varying, on the average, from 6 to 24 % [Korenchevsky *et al.*, 1933, 1, p. 562]. In the present experiments "Pr" alone (Table VI "% change on controls") produced a similar small, but constant increase, varying from 10 to 15 %, which was further slightly increased by the addition of injections of oestrin (last column, Table VI). The average figures for this additonal effect of oestrin were practically identical in the "Pr" experiments (+14 %, Table VI), and in the "H" experiments (+15 % and +14 %, Table VII).

It must be remembered that after castration a small decrease in the weight of the kidneys was found in male rats [Korenchevsky and Dennison, 1934, 1]. Therefore, in spite of the small effects, the constancy of the results obtained with various arrangements of the experiments indicates that both male and female sexual hormones have a slight but specific stimulating effect on the weight of the kidneys of castrated rats.

*Effects on the thyroid and adrenals.* The addition of oestrin caused no changes in these endocrine organs.

*Effect on the liver.* Although the addition of oestrin to testicular hormone had no effect on the liver, we should like here to emphasise an important influence of "Pr" testicular hormone preparation injected alone (Table VI), namely the increased weight of the liver, shown both in the actual weight (on the average +41 %) and in the weight calculated per unit of body weight (on the average +19 %). These results are exactly the same as those published earlier [Korenchevsky *et al.*, 1933, 1; p. 562], in which the average increase in the weight of the liver (per unit of body weight) was shown to be from 17 to 25 % when castrated rats were injected with "Pr" for 21 days. On the other hand we obtained a decrease in the weight of the liver after the castration of male rats [Korenchevsky and Dennison, 1934, 1].

We have not sufficient data to come to any conclusion with regard to the preparation "H," since the control uninjected groups (not included in the tables) in two litters Nos. 15 and 16 had pneumonia (which reduces the weight of the liver), leaving only one healthy uninjected control in the third litter. In this last litter, however, there was no increase in the weight of the liver after the injection of "H" alone, or in combination with oestrin. It would therefore be interesting to repeat the experiments with "H" preparation, in order to decide whether the absence of influence of "H" was accidental, or indicated a lack in "H" of substances stimulating the liver of castrated rats.

It is possible from these experiments, however, to suggest that testicular

hormone, and not oestrin, has a stimulating effect on the liver. This is in agreement with the results published in the previous paper showing the absence of a stimulating effect on the liver of injections of oestrin alone.

## Discussion.

### *The assay of male sexual hormones.*

The results of our experiments corroborated the result of Freud [1933], that oestrin, when injected simultaneously with testicular hormone, usually causes a considerable increase in the effect of the latter on the weight of the seminal vesicles.

Our results also showed that the effects of these combined injections on the weight of the prostate, penis and preputial glands were small, or absent, in 7-day experiments (the period of injection we proposed for the assay of testicular hormone). When the injections were made for 21 days, the effects were larger, only with the preparation "Pr."

We proposed, for the assay of testicular hormone, to use the percentage change in the weight of the prostate with seminal vesicles weighed together, 40 % increase being considered equivalent to 1 R.U. [Korenchevsky, 1932, 1, 2; Korenchevsky *et al.*, 1932].

It can be seen from Tables II and III that, although the differences were not very large in most litters with "Pr" and in all litters with "H," the addition of oestrin caused a smaller percentage increase in the weight of the prostate alone than in that of the prostate weighed together with the seminal vesicles. That the effect of the addition of oestrin is comparatively small, when these glands are weighed together, is chiefly due to the fact that the weight of the seminal vesicles is only from 1/4 to 1/3 that of the prostate and that to a considerable extent, therefore, the weight of the prostate masks the changes in the weight of the seminal vesicles. However, taking into consideration that oestrin in combination with testicular hormone may cause a considerable increase in the weight of the seminal vesicles and produces only a very small change in the weight of the prostate, it would be more cautious to use the change in weight of the prostate alone for the assay of testicular hormone.

At the same time, a separate weighing of the seminal vesicles and a comparison of the changes obtained with those on the prostate alone may give a rough indication of the degree of purification of the testicular hormone from oestrin or oestrin-like substances, since the degree of increase of the seminal vesicles is usually much greater if oestrin is present in the testicular hormone preparation. While the addition of oestrin to the preparation "H" made the difference between the reaction of prostate and that of seminal vesicles quite obvious (Table III), this was not always so with the preparation "Pr" (Table II, litter 1). The occurrence of such large variations makes the explanation of the reaction of the seminal vesicles more difficult and probably will render these organs in castrated rats less satisfactory for the purpose of assay.

It would only be possible to work out an accurate relationship between the increase in weight of the prostate and that of the seminal vesicles on injecting testicular hormone alone or mixed with oestrin, by using pure testicular hormone, which is at present not available, and pure oestrin.

On the basis of the results obtained in the present paper, however, for the assay of the male hormone, we suggest the use of the percentage changes in the weight (per unit of body weight) of the prostate alone and not of the combined

weight of the prostate with seminal vesicles, which we suggested in our previous paper [Korenchevsky *et al.*, 1932].

This alteration raises the question as to whether, in the case of purified testicular hormone preparations (in the absence of oestrin), the number of R.U. as assayed on the prostate alone will differ from that as assayed on the prostate and seminal vesicles together. The following figures are an example of the average percentage changes, as compared with the uninjected control littermates, in the weight of the prostate alone and of the prostate and seminal vesicles of castrated rats, injected for 7 days with different doses of "H" or "Pr" in 11 litters used for the assay of these preparations. The two lower lines are obtained by dividing percentage change by 40 (see definition of R.U. [Korenchevsky *et al.*, 1932]).

| | No. of litter ... | I | II | III | IV | V | VI | VII | VIII | IX | X | XI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % increase in | Prostate + seminal vesicles | 219 | 54 | 76 | 80 | 65 | 69 | 152 | 93 | 124 | 95 | 88 |
| | Prostate alone | 217 | 64 | 77 | 78 | 73 | 79 | 142 | 81 | 129 | 94 | 103 |
| No. of R.U. given by | Prostate + seminal vesicles | 5·5 | 1·4 | 1·9 | 2·0 | 1·6 | 1·7 | 3·8 | 2·3 | 3·1 | 2·4 | 2·2 |
| | Prostate alone | 5·5 | 1·6 | 1·9 | 2·0 | 1·8 | 2·0 | 3·6 | 2·0 | 3·2 | 2·4 | 2·6 |

A comparison of these figures shows that, in the case of sufficiently purified male hormone preparations, the number of rat units is the same or nearly the same when using the weight of the prostate alone or together with the seminal vesicles.

Again, a striking similarity in the results of the percentage increase in the prostate + seminal vesicles and in the prostate alone after 21 days of injection is seen in Table IV (see column "% change on controls, Pr 1·5").

Moreover, when assaying various batches of "Pr" and one batch of "H," we found that the strength of these batches in our proposed rat units was the same as the value given in capon units, *i.e.* 1 C.U. was about equal to 1 R.U. [Korenchevsky *et al.*, 1932; 1933, 2]. The percentage changes in the prostate are thus suitable for the assay of that male hormone which produces comb-growth in capons, for which purpose many investigators have found the comb-growth method to be satisfactory.

We have found in the previous papers, however [Korenchevsky *et al.*, 1933, 1, 2], that comparing the number of C.U. with the number of R.U. (as defined by us) the whole sexual activity in some preparations was about double that of the comb-growth activity. We should, therefore, have failed to recognise half the sexual activity of some preparations if we had not used our method of assay.

Thus the results obtained seem to justify the use of this method of assay, since it gives at least two data at the same time: (1) on prostate alone, an assay of the hormone promoting comb-growth, and (2) on prostate with seminal vesicles (weighed together), an assay of the whole male sexual activity of the preparation. In addition, the percentage changes in the seminal vesicles alone may give supplementary information as to the presence of oestrin, or oestrin-like substances, in the preparation.

It seems to us that the changes in weight of the seminal vesicles alone cannot be taken as a measure of the whole male sexual activity, since their exceptionally great reaction to oestrin and oestrin-like substances is not shown by the remaining male secondary sexual organs.

On the other hand, the change in the weight of the seminal vesicles + prostate gives a rough representation of the true result of the whole sexual activity, since the changes due chiefly to oestrin-like substances (seminal vesicles) and the changes due to the comb-growth-promoting substance (prostate) are given in their natural relationship. In addition, from the results of some of our present experiments, it would seem that the reaction of the seminal vesicles alone does not always give results which are regular enough for assay purposes in castrated rats.

We give as an example the following experiment taken at random and performed on a litter of castrated rats in the assay of one batch of preparation "H" (changes per unit of body weight):

| | | 1·5 "H" alone | 1·5 "H" + 60 oestrin |
|---|---|---|---|
| % change in weight of | Prostate + seminal vesicles | 76 | 133 |
| | Prostate alone | 77 | 92 |
| | Seminal vesicles alone | 67 | 300 |

Assuming the effect of oestrin on the prostate to be comparatively negligible for assay purposes, the strength in the preparation of the hormone promoting comb-growth was between 1·9 and 2·3 R.U. (77/40 or 92/40); the whole sexual activity of the preparation "H" injected alone was 1·9 R.U. (76/40); and that of "H" injected with oestrin 3·3 R.U. (133/40). The presence of the "oestrin-like substance" is suggested by the very large difference in the percentage increase of the seminal vesicles alone and of the prostate alone; and by a considerable increase in the number of R.U. of the "whole sexual" activity as compared with "comb-growth" activity. It should be noted that for our method of assay an average of the results of at least 3 litters is used.

*On the manifold function of the sexual hormones in males.*

Korenchevsky [1930], from the results of his experiments on castrated and cryptorchid rats, suggested that the male gonads produce at least two hormones, both of which influence the secondary sexual organs. The results of the present experiments indicate a similar conclusion, since both testicular hormone and oestrin have a stimulating effect on the secondary sexual organs.

In the paper already mentioned, as well as in a later paper [Korenchevsky and Dennison, 1934, 1], it was shown that in male rats castration produces changes in many non-sexual organs and functions: a decreased gain in body weight, increased fat deposition, slight atrophy of the thyroid, liver and kidneys, delay in involution of the thymus and hypertrophy of the adrenals and hypophysis. On the other hand, the results in our previous and present experiments show that testicular hormone preparations cause a return towards normal of the changes in the thyroids, adrenals, thymus, liver and kidneys and improved the gain in body weight but do not influence the hypophysis or decrease the deposition of fat. This of course only refers to the purified preparations which we were using and not to a pure testicular hormone.

In castrated male rats, oestrin produced in addition to the changes in the secondary sexual organs which have been mentioned above, a decrease in body weight, hypertrophy of the hypophysis, acceleration in the rate of involution of the thymus and possibly had a slightly (but not constant) stimulating effect on the kidneys and spleen.

Thus, on the basis of our experiments on the effects of castration and of injections of testicular hormone and oestrin, alone or combined, these two hormones cannot be regarded as sexual hormones only, but as hormones having

manifold functions, of which one is a sexual function. Even before the completion of the histological investigation such a conclusion seems to us to be justified.

Before drawing any definite conclusions as to the influence on the catabolic processes, which from our results also seems to be present, it is necessary to carry out more numerous and direct experiments, including investigations of the gaseous, nitrogenous and mineral metabolism.

SUMMARY.

1. Experiments were performed on 73 castrated rats belonging to 16 litters and injected for 7 or 21 days with preparations of testicular hormone, alone or in combination with oestrin.

2. The comb-growth method does not assay the whole sexual activity of male hormone preparations, while this is given by the assay on the prostate with seminal vesicles of castrated rats.

3. The addition of oestrin to testicular hormone injections greatly increased the weight of the seminal vesicles (especially with one of the preparations) and (more particularly in the 7-day experiments) caused only a slight, if any, increase in the weight of the prostate, penis and preputial glands.

4. An alteration in the method of assay previously described and in the definition of the rat unit is therefore suggested; namely, that the percentage change in weight of the prostate alone shall be used for the assay of the hormone "promoting comb-growth," and that of the prostate and seminal vesicles weighed together for the assay of "the whole male sexual activity" of the sexual hormone preparations. For this latter assay the changes in the weight of the seminal vesicles in relation to the changes in the weight of the prostate are also important.

5. This alteration in the method does not cause any significant change in the number of R.U. in purified testicular hormone preparations, but eliminates those produced by additional sexual activity (*e.g.* oestrin) in less purified preparations.

6. The rat unit suggested is the minimum daily dose, which when injected twice a day in half doses, during 7 consecutive days, into at least three litters of rats castrated before the 30th day after birth, will produce, on the average, an increase of 40 % in the weight of prostate alone (for the assay of "comb-growth hormone") or in the weight of the prostate with seminal vesicles (for the assay of the "whole male sexual activity") as compared with the average weight of the respective organs in the uninjected litter-mates. The rats for assay should not be used earlier than 30 days after castration.

7. The reasons for not using the changes in weight of the seminal vesicles alone for the assay of the "whole male sexual activity" of the preparations were discussed.

8. The male and female hormones in the male organism are not sexual hormones only, but hormones with manifold effects on other important functions and organs.

9. For ascertaining the other properties of testicular hormone preparations, in addition to the assay of sexual activity, it is most suitable to use rats which have been castrated for not less than 70 days and injected with suitable doses for not less than 21 days.

10. The effect of testicular hormone of increasing the weight of the liver and kidneys (which has been previously described) was corroborated in these experiments.

11. Besides the effect on the sexual organs, the addition of oestrin to testicular hormone injections was followed by a decreased gain in body weight and fat deposition, an acceleration in the speed of involution of the thymus, an increased hypertrophy of the hypophysis and a slight increase in the weight of the kidneys.

12. Some differences were noted between the effects of two preparations of testicular hormone when used in combination with oestrin injections.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this research and to them our thanks are due.

REFERENCES.

Freud (1933). *Biochem. J.* **27**, 1438.
Korenchevsky (1930). *J. Path. Bact.* **33**, 607.
—— (1932, 1). *Biochem. J.* **26**, 413.
—— (1932, 2). *Biochem. J.* **26**, 1300.
—— and Dennison (1934, 1). *J. Path. Bact.* **33**, 231.
—— —— (1934, 2). *Biochem. J.* **28**, 1474.
—— —— and Kohn-Speyer (1932). *Biochem. J.* **26**, 2097.
—— —— —— (1933, 1). *Biochem. J.* **27**, 557.
—— —— —— (1933, 2). *Biochem. J.* **27**, 778.
Moore and Price (1932). *Amer. J. Anat.* **50**, 13.
Schoeller and Gehrke (1933). *Arch. Gynaek.* **155**, 234.

# CXCVIII. THE METALLIC CONTENT OF TISSUES FROM GOAT FOETUSES AND KIDS.

By HUGH RAMAGE.

*From Ridgemont, Carrow Hill, Norwich.*

(*Received July 3rd, 1934.*)

Tissues from human foetuses were examined for mineral content by Sheldon and Ramage [1931; Ramage *et al.*, 1933], and it is desirable that the method used by them should be extended to other foetuses in order to make a comparative study of the quantities of metals present at various stages of development and to learn something further of the functions of the common metals. The tissues of the goat foetuses and kids forming the subject of the present paper were obtained through the kindness of Prof. J. Barcroft, Cambridge, and Prof. H. A. Harris, University College, London, who studied the animals before dissection of the tissues. The specimens included the kidneys, spleen, liver, meconium, gall bladder and bile from each of eight animals, the ages and approximate weights of which were as follows:

| | No. | Weeks | Days | g. | |
|---|---|---|---|---|---|
| Foetus | 18 | 12 | 5 | 240 | A twin |
| ,, | 19 M | 14 | 5 | 562 | ,, |
| ,, | 19 F | | | 538 | ,, |
| ,, | 21 | 19 | 3 | 833 | One of triplet |
| ,, | 22 | 20 | 4 | 2600 | A twin |
| Kid | 23 | 21 | 4 | 2300 | ,, |
| ,, | 24 | | | 1850 | ,, |
| ,, | 20 | 22 | 2 | 4800 | ,, |

The gall bladders were tied up and sent green, the other tissues were partially dried and, on receipt here, each was powdered in an agate mortar and dried in a steam-oven to constant weight. The gall bladders were washed on the outside with water, dried on filter-paper, slit open and the bile removed. Weighed or measured quantities of the bile were put on pieces of ashless filter-paper and dried ready for spectrographic analysis by the flame method [Ramage, 1929]: the bladders were dried and powdered.

The wet weights of the bodies and the livers and the dry weights of the other tissues were taken and plotted against the ages. The most noticeable feature observed was the rapid growth of the kidneys up to 14 weeks 5 days, the kidney then weighed 82 % of the kidney of kid No. 20, whereas the body was only 11·5 %, the spleen 32 %, and the liver 30 %, of the weights of the body, spleen and liver of that kid, respectively.

The mineral content of the dried tissues was determined by burning 0·025 g. of the powder of each in rolls of ashless filter-paper in an oxy-coal gas flame before the slit of a spectrograph as described in previous papers. Smaller quantities only of some of the gall bladders and bile were available, and in those cases the whole of the specimens were used in the analyses. The elements studied were potassium, rubidium, magnesium, calcium, manganese, copper and iron.

The rate of growth of the foetuses and kids varied from case to case; No. 21, for instance, one of a triplet, was much below par in almost every particular studied, both in weight and percentages of the mineral contents. While, therefore, it would be unwise to attempt to draw deductions from minute differences, there are points of a more general character which may be regarded as typical of average development.

There is distinct evidence of a marked shortage of mineral elements, especially copper, potassium, and, to a less extent, of iron towards the end of foetal life. It is known that in the newly born child, calf and piglet, the percentages of copper in the livers are six to ten times as high as in the mothers' livers, but in these goat foetuses and kids the percentage was only 0·002 in Nos. 21, 22, 23 and 24, whereas it was 0·02, 0·015 and 0·01 in Nos. 19 M, 19 F, and 20 respectively. The percentage of copper in the human liver nearly doubles during the last three months of foetal life, rising from 0·016 to 0·03 [Ramage *et al.*, 1933].

The shortage of metals was seen in every tissue except the kidney in which the iron rose from 0·012 to 0·03 %, and, as noted above, the growth of the kidney is very slow after about the fourteenth week.

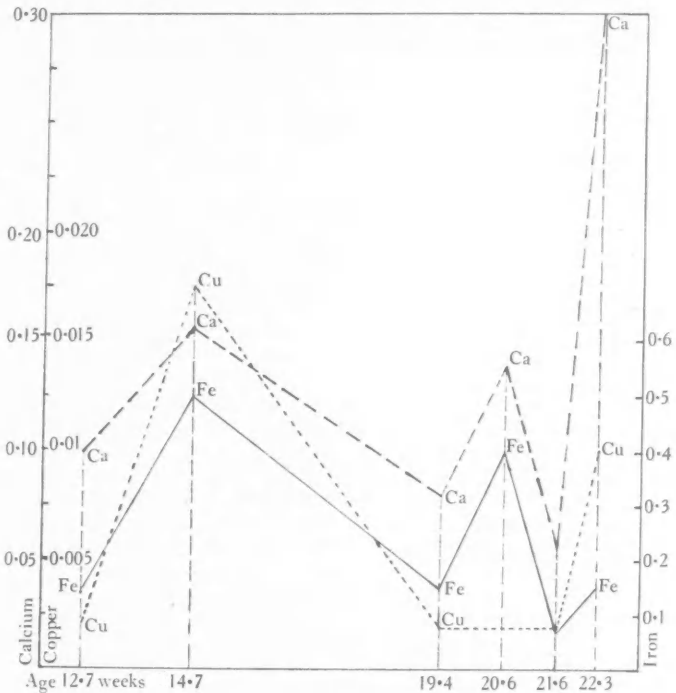



Fig. 1.

The livers and meconia of the foetuses Nos. 19 M and 19 F were remarkable, as the percentages of iron and copper in the livers were higher than in any other of all the tissues analysed and the meconia contained more copper than that of

any other foetus or kid. Only three meconia contained manganese. No. 19 M contained 0·006, No. 19 F contained 0·001 and No. 20 contained 0·0006 %. These features do not appear to be accidental. The kid No. 20 lived for a day, receiving some milk, and the "meconium" contained far more calcium and less magnesium than any other.

There was distinct evidence also of correlation between iron, calcium and copper in the livers (see Fig. 1), as was found to be the case in children's livers [Ramage *et al.*, 1933], the only exception being that copper in No. 22 was relatively low, but this again may be accepted as further evidence of the shortage of copper towards the end of foetal life in these goats.

The human infant, the calf and the piglet are all born with a reserve stock of copper in their livers but these kids had no reserve, the percentage in fact being extremely low.

Manganese, as in children's livers, followed an independent line, the percentage in the livers and gall bladders was highest about birth, and there were merest traces in the kidneys of Nos. 23 and 24, but in no other kidneys, and no manganese was detected in the spleens.

Grateful acknowledgment is made for grants from the Government Grant Committee of the Royal Society towards the cost of apparatus, *etc.*

REFERENCES.

Ramage (1929). *Nature*, **123**, 601.
—— Sheldon and Sheldon (1933). *Proc. Roy. Soc. Lond.* B **113**, 308.
Sheldon and Ramage (1931). *Biochem. J.* **25**, 1608.

# CXCIX. RICKETS IN CHICKENS, WITH SPECIAL REFERENCE TO ITS NATURE AND PATHOGENESIS.

By JOHN POOL McGOWAN and ARTHUR RAYMOND GORDON EMSLIE.

*Aberdeen.*

(*Received June 27th, 1934.*)

Much has been written on "leg weakness" in chickens, a subject of varied aetiology and pathology although the term is often employed as synonymous with rickets. One is not here concerned with those varieties included under this designation which are associated with lack of vitamin B, absence of some factor in milk [*vide* Hogan *et al.*, 1925; 1928; Norris *et al.*, 1930; 1931; Bethke *et al.*, 1931], neurolymphomatosis or pellagra. Perosis or "slipped-tendon," however, from the special circumstances in regard to Ca and P metabolism surrounding its genesis, merits detailed remark. There is a voluminous literature on this subject. Amongst recent papers are those by Titus [1932]; Holmes *et al.* [1933]; Parkhurst and MacMurray [1933]; Branion [1933] and many others. Two characteristics of the condition are worthy of note: firstly, that cod-liver oil is powerless to prevent or cure it; and secondly, that nothing abnormal is to be found on histological examination of the bones. As regards aetiology, it is almost universally agreed that the most commonly associated precursor of the condition is the presence of large amounts of $Ca_3(PO_4)_2$, as bone meal, in the ration. This point will be discussed later, but meantime it may be mentioned that elsewhere [McGowan, 1933, 2] evidence has been adduced to show that metabolism of Ca and P is much less efficiently carried on when these elements are fed in chemical union with one another. The alternative, of course, is to feed the Ca as the salt of some other acid radical than $P_2O_5$, while the P may be supplied in a "delayed-action" form as food-phosphorus which requires to be broken up into simpler chemical form by digestion before it is absorbed. In this case it will happen that the Ca is for the most part dissolved out and absorbed before the P is in a form to unite with it. The prevention of perosis [Titus, 1932] by the administration of rice bran, rich in organic P of the "delayed action" type, lends support to this view. Melby [1933], by statistical analysis of experimental results obtained by various authors, also showed that a high P content[1] of the ration was associated with a high percentage occurrence of perosis.

With regard to rickets, the main subject of this communication, some preliminary considerations require emphasis. It is a singular fact that histological examination of the bones has played no part whatever as a routine procedure and check in experiments on the production of rickets in chickens by means of various diets. Indeed, there are very few contributions at all in the literature dealing with the morphological appearances in the bones, and such as exist are mainly for the purpose of settling some minor disputed point, or *ad hoc* and of the nature of investigations in pathology. One mentions, more or less in the

[1] Excess P had been given as $Ca_3(PO_4)_2$.

former context, the work of Hart *et al.* [1925], Hughes [1924], Pappenheimer and Dunn [1925] and Hughes and Titus [1926]. Pappenheimer and Dunn [1925] maintained that the condition present in the bones of chickens fed on the usual "rickets"-producing ration (that of Hart [1924])[1] is not rickets and would seem effectively to have disposed of the allegations to the contrary of the other investigators mentioned. Pappenheimer and Dunn, however, while describing the condition present do not venture on its classification on a pathological basis. At a later date, Nonidez [1928] made a more definite contribution to this subject. He pointed out that the bones of the chick on hatching were in a much earlier stage of development than those of the mammal at birth. He alleged that their bones reached the stage of those of the new-born mammal only after five weeks of post-hatching life, a view which will afterwards be shown to be incorrect. On a certain special diet, his experimental chickens failed to grow and at six weeks most of them were dead, while their bones, he stated, showed the changes already described by Pappenheimer and Dunn. The addition of semisolid buttermilk to this ration, together with exposure of the birds to sunlight, permitted the chickens to live longer and, at the same time, to exhibit better growth and bone development, though these apparently were not as yet optimum[2]. Chickens were now reared for the first 4 weeks on this modified ration together with exposure to sunlight, and thereafter, the semisolid buttermilk having been withdrawn from the ration, were kept indoors and away from sunlight. Birds so treated, he alleged, developed rickets, and this of a type comparable with that of mammals: but it must not be forgotten that in his diagnosis of rickets he stresses the existence of enlargement of the ends of the bones, beading of the ribs, *etc.*, as an essential feature. The presence of such lesions in his later experiments as compared with his earlier, can, however, be attributed to the greater age to which the chickens of the former lived; while it will be shown later that such enlargements occur as frequently and as characteristically in osteoporosis as in rickets, and that sunlight and vitamin D have equal effects in preventing both. Other points in his claims are, moreover, not quite clear. Thus a ration which, when fed from hatching onwards, produced (as Nonidez admits) Pappenheimer and Dunn's non-rickety condition, is alleged to be able to bring about true rickets when fed to healthy chickens over six weeks of age, a claim which appears not to be fully substantiated by the illustration (Fig. 1) accompanying his paper. This photograph depicts an appearance more in keeping with a modified osteoporosis, although it is admitted that a band, which might be interpreted as a rachitic metaphysis, is present and is designated as such in the text. He finds, in addition, enlargements of the parathyroids and a lowered Ca content of the blood of his birds, conditions more in harmony with the existence of osteoporosis, as will afterwards be pointed out. That sunlight prevented and cured the condition elicited by him in his experiments has no significance either way, as it has as great a beneficial influence on certain types of osteoporosis as on rickets.

The histological investigations, therefore, which have been undertaken in the sphere of the Ca and P metabolism of the chicken would seem, so far as they show anything, to have demonstrated the existence of osteoporosis, while nothing of the nature of rickets has with certainty been disclosed. Moreover, they were undertaken without idea of their serving as a check on the results

[1] From personal observations, the condition produced in chickens fed on this ration (yellow maize 97, $CaCO_3$ 2, NaCl 1 part), preventable and curable by cod-liver oil, is osteoporosis—not rickets.

[2] See footnote, page 1507, in reference to this point.

obtained in feeding experiments. The only check that has been consistently and largely applied for this purpose is an estimation of the total ash or the Ca and P in the bones. Unfortunately such a procedure is exceptionally open to criticism, apart from the fact that several authors, such as St John *et al.* [1933] and Bethke and Record [1934], have impugned the accuracy of the test as such. Osteoporosis, a condition differing in every other particular from rickets, resembles it only in having a low ash content in the bones and the swelling of the ends of them already mentioned. Thus, from the experimental results presented below it will be seen that while the tibiae of normal chickens of group BD had 51 % ash at six weeks of age, those of the osteoporotic chickens of groups AD and B at the same age had respectively 36·05 and 41·2 % and those of the rickety chickens of group C 27·8 %. Similar figures were obtained in these groups at 2, 3, 4 and 5 weeks of age[1]. The significance of such results is apparent, and as a sequel to such considerations, the value of much of the previous work on rickets in chickens is incapable of being assessed owing to the lack of certitude regarding the exact condition, osteoporosis or rickets, under observation.

Criticism can also be applied to the voluminous literature that has arisen around the alleged importance in nutrition of the chicken of the ratio of Ca to P in the ration. This is quite apart from the obvious fact that the same ratio may exist between very different absolute quantities of Ca and P. In addition, evidence has been adduced elsewhere [McGowan, 1933, 2] to show that ratio, *per se*, is of no importance and that, apart from a real absolute deficiency of Ca or P in the ration, the only matter of moment in this connection is the percentage content of Ca in the ration in relation to its power of neutralising the HCl of the gastric juice and thereby setting in motion a train of injurious consequences.

## Experimental.

The present investigation deals in the main with the histological changes present in the bones and the implications of such in chickens reared on diets of a nature such as had previously been alleged to produce or prevent rickets, and forms the culmination of a series of observations carried out on this subject since 1926.

The chickens employed were 3-day old White Leghorns. They were reared in brooders inside a house and away from sunlight. The basal diet was as follows:

| | | |
|---|---|---|
| Yellow maize | 75 | parts |
| Dried skim milk | 10 | ,, |
| Dried yeast | 15 | ,, |
| Sodium chloride | 0·5 | ,, |

The six groups (15 birds in each[2]) employed and the CaO and $P_2O_5$ contents of the various rations are given below:

| Group | | CaO % | $P_2O_5$ % |
|---|---|---|---|
| A | Basal | 0·24 | 1·49 |
| AD | Basal + 1 % cod-liver oil | 0·24 | 1·49 |
| B | Basal + 1·2 % $CaCO_3$ | 0·91 | 1·48 |
| BD | Basal + 1·2 % $CaCO_3$ + 1 % cod-liver oil | 0·91 | 1·48 |
| C | Basal + 6 % $CaCO_3$ | 3·6 | 1·48 |
| CD | Basal + 6 % $CaCO_3$ + 1 % cod-liver oil | 3·6 | 1·48 |

[1] The lowest ash percentage (22·6 %) was obtained in the case of an osteoporotic chicken (group AD) placed at 6 weeks into group C (rachitic) for 3 weeks.

[2] Owing to their greater rate of growth, males suffered more severely than the females.

At intervals of a week the chickens were weighed individually, and specimens from each group were removed and killed for the purpose of examination of the bones histologically and with regard to their ash content. The weights of the chickens at 6 weeks, exhibiting as they do the same relative difference between the various groups as had been obtained in the previous four weighings, is first submitted. The figures for group A, basal ration group, are omitted as the chickens in this group were all dead before the expiry of the third week.

Average weight of chickens (g.) of each group at 6 weeks:

| | |
|---|---|
| Group BD | 333 (normal bones) |
| ,, CD | 269 ,, ,, |
| ,, C | 213 (rachitic bones) |
| ,, B | 186 (osteoporotic bones) |
| ,, AD | 107 ,, ,, |

The average weight in group BD—333 g.—is up to the standard of growth for this race of fowl under ideal conditions of food and surroundings (*vide* Charles and Knandel [1928]; also personal observation). The series of body weights given here will be seen by comparison with the preceding table to run parallel with the amount of Ca actually available for ultimate metabolism in the various groups and, in this connection, attention may be particularly directed to the very divergent results as regards mortality, weight and growth in group A (basal) as compared with group AD (basal + cod-liver oil), where the very low Ca in the basal diet is seen to be made in some degree more available in the second group by means of vitamin D. As will also be seen from a comparison of the size of the bone sections in the various groups at 6 weeks of age (*vide* Plate IX), the difference in size of the body as a whole is paralleled by a similar divergence in the size of the bones. Deficiency of Ca (with ample P present) would appear, therefore, to bring about lack of tissue growth and an atrophy and to produce in the bones osteoporosis, as will be seen more fully immediately; while over-abundant Ca (with consequent deficiency of P occurring simultaneously) allows growth, possibly of an exuberant nature, to proceed, which in the bony tissues remains immature and gives rise to rickets, as will be discussed shortly.

As a detailed description of the histological appearances present in the bones is attached to the illustrations accompanying this paper, only the broad general findings will be dealt with here. From the examination made at the end of six weeks, they may be summarised as follows:

| | | | |
|---|---|---|---|
| (1) | Groups BD and CD | (basal + $CaCO_3$ + cod-liver oil) | bones normal (*vide* Plate IX, Figs. 3, 4) |
| (2) | Groups A | (basal) | |
| | AD | (basal + cod-liver oil) | osteoporosis (*vide* Plate IX, Figs. 5, 6) |
| | B | (basal + 1·2 % $CaCO_3$)[1] | |
| (3) | Group C | (basal + 6 % $CaCO_3$) | rickets[2] (*vide* Plate IX, Fig. 7) |

The same results were obtained in the bones from the various groups at the end of 2, 3, 4 and 5 weeks from the commencement of the experiment. Group BD, normal group, in contrast to the view expressed by Nonidez [1928] on this

[1] Somewhat similar osteoporosis with ample CaO in the diet and preventable by the addition of cod-liver oil has been noted in pigs [McGowan, 1933, 3].

[2] The appearances present here differ somewhat, though not in essentials, from those usually found in mammalian rickets, especially in sections of costo-sternal rib junctions. They are simpler in type in the present instance, which would seem to indicate, as the appearances do, that in the latter case complications have arisen owing to movement and fracture and the production of callus [*vide* McGowan, 1924].

subject (*vide supra*), showed the epiphyses fully formed and of "mammalian type at birth" at one week of age (Fig. 2)[1].

Chickens from groups AD and B (osteoporotic groups) and from group C (rickets) when placed at five weeks of age in group BD (normal) showed complete recovery and normal appearance of the bones in a week (Plate IX, Figs. 8, 9, 10). The recovery may actually have been more rapid since specimens for examination were taken only at the expiry of a week.

Chickens from group AD (osteoporotic group) transferred at 6 weeks of age to group C (rickets group) and kept there for 3 weeks showed then a mixture of osteoporosis and rickets, while the ash percentage of the bones (22·6 %) was the lowest obtained in the investigation.

Other findings of importance emerged. Thus, consistently in all the examinations at the various time intervals of a week, the parathyroids in groups A, AD and B (the osteoporotic groups) were found to be enlarged: while in groups BD and CD (the normal groups) and group C (the rickety group), they were normal in size. Doyle [1925], Nonidez and Goodale [1927], Bloom [1932] and Higgins and Sheard [1933] have remarked on the enlargement of the parathyroids in chickens in cases where, however, deprivation of vitamin D was regarded as the causal precursor: but it will be seen from the above classification that this enlargement of the parathyroids has relation rather to deficiency of available calcium in the body. Nonidez [1928] has indeed pointed out what he regards as a significant difference in the reactions produced by rickets in mammals as compared with chickens in that he found, in chickens affected with "rickets," a lowered Ca in the blood, a condition of matters, he remarks, not found in the mammalian disease. In this case, however, all the attendant circumstances indicate that the disease in his chickens was in reality osteoporosis and not rickets; and the results obtained in this experiment support this contention. It was found that, while the normal and rickety groups showed Ca 11–12 mg./100 ml. Ca in the blood-serum, the osteoporotic groups were quite definitely subnormal in this respect, having only 9·5 mg./100 ml. serum. Higgins and Sheard [1933] obtained similar low figures for the serum-Ca (8·55–6·38 mg./100 ml.) by feeding chickens on a diet containing dicalcium and tricalcium phosphates, although, as they remark, the calcium provided in the diet in this way was adequate, a point of interest in connection with the findings obtained in group B (basal + 1·2 % $CaCO_3$) in the present experiment. In their case too, the Ca was rendered available by the administration of cod-liver oil.

Enlargement of the bones, especially of the distal ends of the sternal and vertebral ribs and of their heads and tuberosities, was found to a comparable degree in the osteoporotic and rachitic groups, if due allowance be made for the age and growth of the individual bird. This is contrary to the view expressed by Nonidez (*vide supra*).

Observations were carried out on the point raised by Nonidez [1928] of the difference in histological appearance of the bone lesions according to whether the chickens were put on experiment at once on hatching or only after 5 weeks of post-hatching healthy growth. Chickens from groups BD and CD (normal with normal epiphyses) were, at the age of 5 weeks, transferred to group C (rachitic group) while, at the same time, others from the same normal groups were put into group B (osteoporotic group). The conditions produced in the bones, after a suitable lapse of time following such transference, were the same in type as

[1] This indicates that Nonidez's ration, even when fortified with dried buttermilk, was far from being a good one, and that indeed from the outset normal calcification did not occur in his chickens.

those obtained where birds, immediately on hatching, were placed in groups C and B, and were modified only to a very slight degree by the more developed condition of the epiphyses. The rachitic ration still produced unmistakably rachitic, and the osteoporotic ones, osteoporotic lesions (Plate IX, Figs. 11, 12).

## Discussion.

The results obtained in this experiment will be discussed in relation to the theory elaborated elsewhere regarding the nature of vitamin D action [McGowan *et al.*, 1931; McGowan, 1933, 1, 2, 4]. The essential action of vitamin D, according to this theory, is supposed to be two-fold in nature: firstly, the setting free of nascent phosphoric acid from the phospholipins of the tissues and, secondly, on account of this, the production of an acidotic tendency of the body, which, acting in the blood, prevents the precipitation of Ca as calcium phosphate and favours its circulation in soluble form as the chloride. This acid condition also favours the secretion of HCl in the gastric juice, with resultant benefit to the absorption of Ca and P.

The results obtained in group AD (basal + 1 % cod-liver oil) would appear to show that, of the two actions of vitamin D just referred to, the liberation of phosphoric acid is, as indeed one would expect, the more fundamental and important. In the group under survey, the Ca, present in deficient amount in the ration but presumably all absorbed, is, in contradistinction to what happens in group A, by the intervention of vitamin D made available and anchored in the body for further utilisation. The beneficial influence of vitamin D in this case, however, is circumscribed by Ca being present in too small quantity; hence the comparatively unfavourable results obtained in this group.

The results of the later groups show how markedly cod-liver oil, when Ca is present in adequate amount, can compensate for the unsuitable form of presentation (as $CaCO_3$) or over-abundant supply of this substance. Under favourable conditions of supply of Ca and P, as in milk, mammals can be reared without the aid of vitamin D when the percentage of CaO and $P_2O_5$ is as low as 0·4 [McGowan, 1933, 3]. Figures are thus forthcoming for a minimum concentration of these substances in the ration for healthy growth in mammals in the absence of vitamin D. Attempts have been made to arrive at similar figures for the rations of chickens; but the value of any so far obtained is discounted by the fact that vitamin D, in some form or other, was operative in every case at the same time.

The body of the chicken (differing in this respect markedly from the mammal), when hatched from the shell, owing to Ca during incubation being obtained only in sparse amounts from the egg-shell, is greatly lacking in Ca and its bones are in a markedly osteoporotic condition (Plate IX, Fig. 1). It is quite evident, therefore, that, in regard to Ca metabolism, the findings derived from mammalian studies cannot be transferred bodily to avian conditions. The distinction already pointed out by Nonidez [1928] in regard to the histology of the bones is concrete evidence of this.

While the ordinary diet of chickens (consisting as it does for the most part of grains) supplies abundant $P_2O_5$ and this in "delayed-action" form, it is quite otherwise with regard to calcium, which is notoriously lacking in such food. It is highly probable that the $P_2O_5$ requirements of the birds will be completely met from the food without recourse to vitamin D being required. Calcium, however, in some form or other, must be added to such a ration, and only further investigations directed to this point will be able to elucidate whether the addition of some certain Ca-salt, in an optimum amount alone and without the aid of

vitamin D, will suffice for healthy growth or whether it is essential that vitamin D be added at the same time. Observations already carried out in connection with this matter, show that Ca, added to the present basal experimental ration as calcium chloride or lactate in amount supplying as much as 1 % CaO, failed to prevent osteoporosis. At 4 weeks of age, the birds on this modified ration were only half the weight of birds of the same age in group BD (basal + 1·2 % $CaCO_3$ + 1 % cod-liver oil (0·91 % CaO)), while their bones were in a condition of advanced osteoporosis.

The compensatory action of vitamin D, in cases where ample Ca has been added to the ration, is not only undeniable but considerable. In all likelihood, it depends in this case to a great extent on its acid-producing powers: but, of course, the fixing power on calcium of nascent phosphoric acid must not be lost sight of. As the circulating blood can hold only a limited amount of Ca in solution, if, under ordinary circumstances, large quantities of Ca (as $CaCl_2$) are absorbed rapidly from the food into the circulation, much of it will be excreted immediately in order that the blood composition, *qua* Ca, may be kept within normal limits; and in the alkalotic tendency produced by the administration of large amounts of $CaCO_3$, the precipitation and consequent loss of Ca from the blood will be intensified. *Per contra*, an acidotic tendency due to vitamin D will favour its retention in the circulation. It will also render easier the secretion of acid in the gastric juice, with consequent better solution and absorption of Ca. Both these factors would seem to play an important rôle in assisting Ca metabolism and fixation in the body and in transforming the osteoporotic condition, present in group B for instance, into the normal one found in group BD.

The same factors would seem to be active in bringing about the more or less normal conditions present in group CD as contrasted with the rickety ones in group C. An important distinction, however, has to be made between the fundamental activities operative in the two sets of circumstances. In the set at present under discussion, the phosphoric acid-generating power of vitamin D would seem to play a relatively greater part[1] while the increased acidity of the stomach would seem to effect its purpose in a somewhat different way. In reference to the last point, the $CaCO_3$, present in great excess, is rapidly rendered soluble by the more abundant HCl and removed by absorption into the blood, so that it is no longer able by its presence in the intestine to interfere with and render insoluble as $Ca_3(PO_4)_2$ the products of the "delayed-action" phosphorus when they are set free by digestion.

In the young fowl, therefore, osteoporosis on the one hand as the result of too little Ca[2] and rickets as the result of too much Ca on the other, are alike prevented by the presence of vitamin D. In all probability, this condition of matters arises from the osteoporosis with which the chicken starts life, coupled with the extensive Ca metabolism which consequently has to be carried through in a very short period of time to make up the leeway.

Less stringent conditions with regard to Ca exist in the early life of the young mammal, in which, under ordinary conditions, rickets (due to a relative deficiency of P) is the more common occurrence. Rickets, when it occurs in birds, is usually brought about artificially as the result of an endeavour to overcome, by generous administration, the initial failing of lack of Ca with its consequent osteoporosis; while in mammals, the condition is frequently initiated or, when

[1] By making phosphoric acid in a nascent form circulate more abundantly in the body and also by setting it free actually in the cells where calcification has to take place.

[2] Although ample Ca is present in the food.

already present, aggravated through a similar administration of calcium, based on a misunderstanding of its essential pathogenesis.

It is perhaps not without significance that several authors [Payne *et al.*, 1932; Titus, 1932; Parkhurst and MacMurray, 1933] have laid emphasis on the point that the administration of large amounts of $CaCO_3$ in the ration does not lead to perosis (rickets is not produced in these cases as vitamin D is administered at the same time), but that the feeding of large quantities of $Ca_3(PO_4)_2$ along with vitamin D, in an endeavour to prevent rickets, is very prone to do so. This would seem to indicate some recondite interference with phosphorus metabolism, possibly, as already suggested, related to the fact that Ca and P, when joined in chemical union, are relatively unsuited for tissue metabolism.

## Summary.

It is very unlikely, owing to the type of food usually given, that chickens will suffer from a deficiency of $P_2O_5$ but almost certain that Ca will be lacking.

This latter deficiency is exaggerated and aggravated by the osteoporotic condition of the body of the chicken on hatching, a circumstance which necessitates a very active Ca metabolism to meet it. Vitamin D has only a very slight influence in compensating for this lack of Ca.

In the usual amended diet of chickens, large quantities of Ca, generally as $CaCO_3$, are incorporated but, owing to the alkalosis induced by such foods, the Ca is not absorbed and metabolised, and the condition of osteoporosis, already present, persists and is exaggerated.

It is doubtful whether, even if regard be had to the exact chemical combination of Ca given and the amount of it supplied, Ca in itself as such and without vitamin D could be administered so as to avoid this complication. This point can only be settled by further experimentation, which has already been commenced.

Administered in amounts larger than those associated with the appearance of osteoporosis, Ca, by interfering with the absorption and metabolism of P, produces rickets.

Vitamin D by its fundamental action, working along different lines in the two conditions just mentioned, prevents, on the one hand, the occurrence of osteoporosis, due to deficiency of available Ca, and, on the other, rickets, due to P deficiency.

An important precursor of the condition, perosis, is the feeding of large quantities of CaO and $P_2O_5$ in chemical union with one another.

## REFERENCES.

Bethke and Record (1934). *Poultry Sci.* **13**, 29.
—— —— and Kennard (1931). *Poultry Sci.* **10**, 355.
Bloom (1932). *Proc. Soc. Exp. Biol. Med.* **29**, 860.
Branion (1933). *Poultry Sci.* **12**, 335.
Charles and Knandel (1928). *Penn. Agric. Exp. Sta. Bull.* **218**, 3.
Doyle (1925). *Science*, **61**, 118.
Hart (1924). *J. Biol. Chem.* **60**, 341.
—— Steenbock and Lepkovsky (1925). *J. Biol. Chem.* **65**, 571.
Higgins and Sheard (1933). *Anat. Rec.* **56**, 395.
Hogan, Guerrant and Kempster (1925). *Mo. Agric. Exp. Sta. Bull.* **81**.
—— Shrewsbury and Kempster (1928). *J. Agric. Res.* **37**, 115.
Holmes, Piggott and Moore (1933). *Poultry Sci.* **12**, 356.

Hughes (1924). *Science*, **59**, 213.
—— and Titus (1926). *J. Biol. Chem.* **29**, 289.
McGowan (1924). *J. Path. Bact.* **27**, 409.
—— (1933, 1). *Biochem. J.* **27**, 943.
—— (1933, 2). *Biochem. J.* **27**, 934.
—— (1933, 3). *Brit. Med. J.* ii, 599.
—— (1933, 4). *Brit. Med. J.* ii, 894.
—— Cunningham and Auchinachie (1931). *Biochem. J.* **25**, 1295.
Melby (1933). *Poultry Sci.* **12**, 357.
Nonidez (1928). *Amer. J. Path.* **4**, 463.
—— and Goodale (1927). *Amer. J. Anat.* **38**, 319.
Norris, Heuser and Wilgus (1930). *Poultry Sci.* **9**, 133.
—— —— —— and Ringrose (1931). *Poultry Sci.* **10**, 93.
Pappenheimer and Dunn (1925). *J. Biol. Chem.* **66**, 717.
Parkhurst and MacMurray (1933). *J. Agric. Sci.* **23**, 311.
Payne, Hughes and Lenhardt (1932). *Poultry Sci.* **11**, 158.
St John, Kempf and Bond (1933). *Poultry Sci.* **12**, 34.
Titus (1932). *Poultry Sci.* **11**, 117.

## DESCRIPTION OF FIGURES ON PLATE IX.

In the preparation of the sections, the bones (lower ends of tibio-tarsus) were fixed in 5 % formalin for 24 hours and then cut with the freezing microtome. They were then treated by Von Kossa's silver nitrate method and mounted in glycerol. The sections are in the sagittal plane and the magnification (5 diameters) is the same in each case. There is thus afforded a rough means of comparison as regards growth between the various groups. Calcium deposits are stained deep black.

Fig. 1. Tibia; newly hatched chicken showing *a*, epiphyseal cartilage; *b*, junction of epiphyseal cartilage with shaft cartilage; *c*, cylinder of "foetal" cartilage; *d*, slight deposit of calcium on surface of cartilage column and subperiosteally.

Fig. 2. Tibia; chicken, group BD (basal + 1·2 % $CaCO_3$ + 1 % cod-liver oil); epiphysis fully formed and of "new born mammalian" type after 1 week on this ration: 1 week old: showing *a*, epiphyseal cartilage; *b*, zone of hypertrophied cartilage cells well defined; *c*, spongiosa with numerous well calcified bony trabeculae enclosing normal marrow in their interstices—normal bone.

Fig. 3. Tibia; chicken, group BD (basal + 1·2 % $CaCO_3$ + 1 % cod-liver oil): 6 weeks old: as in Fig. 2—normal bone (lettering as in Fig. 2).

Fig. 4. Tibia; chicken, group CD (basal + 6 % $CaCO_3$ + 1 % cod-liver oil): 6 weeks old: as in Fig. 2—normal bone (lettering as in Fig. 2): *d*, centre of calcification of epiphysis.

Fig. 5. Tibia; chicken, group AD (basal + 1 % cod-liver oil): 6 weeks old, showing *a*, epiphyseal cartilage; *b*, absence of zone of hypertrophied cartilage cells; *c*, "foetal" cartilage (the darkish patches here are due to the presence of folds not to calcification); *d*, very sparse patchy deposit of calcium on free end of this cartilage and subperiosteally; entire absence of calcification elsewhere in this section (although in the actual specimen the background is colourless and without calcified areas, it has been found impossible fully to reproduce this in the photograph)—advanced osteoporosis—(owing to the persisting deficiency of calcium, the intraovular conservative mode of formation of supporting tissues survives to a great extent).

Fig. 6. Tibia; chicken, group B (basal + 1·2 % $CaCO_3$): 6 weeks old: as in Fig. 5—advanced osteoporosis. The great difference between this specimen and Fig. 7 should be noted.

Fig. 7. Tibia; chicken, group C (basal + 6 % $CaCO_3$): 6 weeks old: showing *a*, epiphyseal cartilage; *b*, well developed band of hypertrophied cartilage cells; *c*, wide overgrown band of hypertrophied cartilage cells, arranged in parallel columns, separated by blood vessels: only traces of calcification present; *d*, spongiosa with numerous well calcified trabeculae having normal marrow in the interstices: absence of the osteoporotic changes seen in Fig. 5 and 6—rickets. Note in addition the absence of calcification in the ossification centre in epiphyseal cartilage *e*.

Fig. 8. Tibia; chicken, group AD (basal + 1 % cod-liver oil); placed at 5 weeks of age into group BD (basal + 1·2 % $CaCO_3$ + 1 % cod-liver oil): kept for 1 week on this diet—total disappearance of osteoporosis; bone now normal as in Figs. 2, 3, 4 (lettering as in Fig. 4).

Fig. 9. Tibia; chicken, group B (basal + 1·2 % $CaCO_3$); placed at 5 weeks of age into group BD (basal + 1·2 % $CaCO_3$ + 1 % cod-liver oil): kept for 1 week on this diet—total disappearance of osteoporosis; bone now normal as in Figs. 2, 3, 4 (lettering as in Fig. 2).

Fig. 10. Tibia; chicken, group C (basal + 6 % $CaCO_3$); placed at 5 weeks of age into group BD (basal + 1·2 % $CaCO_3$ + 1 % cod-liver oil): kept for 1 week on this diet—total disappearance of rickets; bone now normal as in Figs. 2, 3, 4 (lettering as in Fig. 4).

Fig. 11. Tibia; chicken, group BD (basal + 1·2 % $CaCO_3$ + 1 % cod-liver oil); placed at 6 weeks of age in group B (basal + 1·2 % $CaCO_3$): kept for 3 weeks on this diet: osteoporosis of marked degree now present, modified slightly from the appearance in Fig. 5 by previous development and persistence of the zone of hypertrophied cartilage: *a*, epiphyseal cartilage replaced by bone consisting of a few slender trabeculae in a background of loose fibrous tissue; *b*, zone of hypertrophic cartilage cells; *c*, subepiphyseal zone with practically no trabeculae and consisting chiefly of a loose fibrous tissue; *d*, zone with few but rather large trabeculae.

Fig. 12. Tibia; chicken, group BD (basal + 1·2 % $CaCO_3$ + 1 % cod-liver oil); placed at 6 weeks of age in group C (basal + 6 % $CaCO_3$): kept for 3 weeks on this diet: shows marked rickets as in Fig. 7: note that there is no removal here of the calcified trabeculae of the normal bone but on the other hand marked proliferation of cartilaginous trabeculae in columnar arrangement from the zone of hypertrophied cartilage cells. These trabeculae remain practically uncalcified. Note also the lack of calcification in the cartilaginous trabeculae in the centre of ossification of the epiphyseal cartilage (lettering as in Fig. 7).

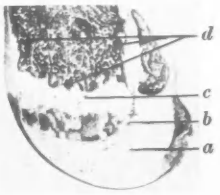
(The osteoporotic changes present at 6 weeks in groups AD and B as also the rachitic ones in group C were present also in specimens from these groups examined at 2, 3, 4 and 5 weeks of age.)

*Addendum* (*August 26th, 1934*). In a previous paper [McGowan, 1933, 3] it was suggested, in view of the remedial action of vitamin D in the disease, that osteomalacia was, in addition to being an acalcicosis, an aphosphorosis. As, however, vitamin D has been shown in the present experiments to have a marked influence on Ca absorption and metabolism; as P is abundant and Ca deficient in the cereal diets which give rise to osteomalacia; and as the addition of Ca is the most important factor in the cure of the condition, these circumstances, together with the histological appearances, render it more probable that osteomalacia is a pure acalcicosis.

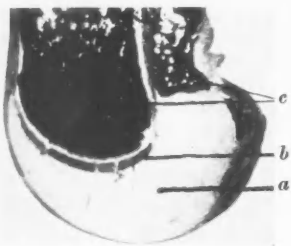
1

2

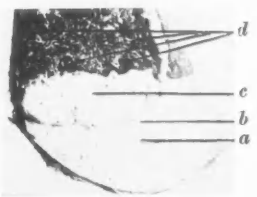
4



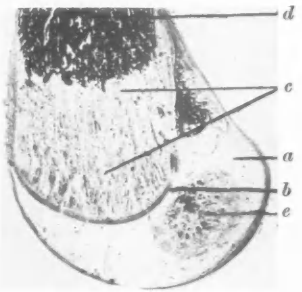

5



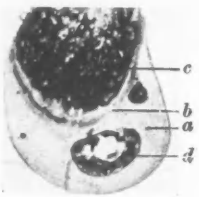

3




6

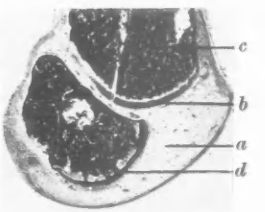



7

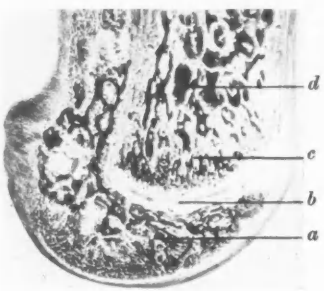



8

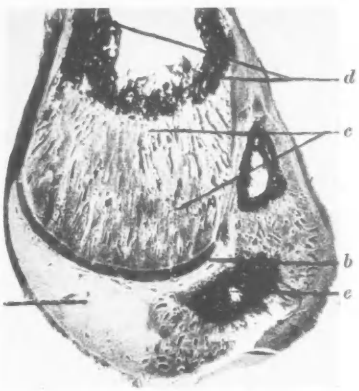
9




10




11




12

# CC. ON THE MECHANISM OF ACIDOSIS IN EXPERIMENTAL URANIUM NEPHRITIS.

By LUCIEN BRULL[1] and CHARLES ROERSCH.

*From the Research Laboratory, Medical Department, University of Liége.*

(*Received June 27th, 1924.*)

In a series of publications concerned with the mechanism of changes in the blood and urine in experimental uranium nephritis, which were studied by the method of double-crossed circulation of Brull [1931], it was stated: (1) that the glycosuria and albuminuria of this nephritis are of purely renal origin [Brull and Fanielle, 1931; Weekers, 1934], (2) that the initial polyuria is due to substances transmitted by the blood [Brull and Fanielle, 1931], (3) that this polyuria is independent of the increased blood-flow which generally occurs [Brull and Roersch, 1933, 1, 2], (4) finally, that the nephritic blood is not immediately toxic for the kidney, since this blood is able to induce considerable hyperactivity in normal kidneys which it perfuses [Brull and Fanielle, 1931].

The present part of our research relates to the mechanism of the profound acidosis noticed by all investigators who have studied the alterations of acid-base equilibrium in acute uranium nephritis.

In a recent series of papers Csapo and co-workers [1932] concluded that the acidosis after ligature of the ureters is due to accumulation of organic acids in the blood. Similar findings were made by Brühl [1933] in human nephritis. The old question of acidosis produced by accumulation of organic acids was thus revived, but we shall not here discuss this matter further [*cf.* Straub and Schleyer, 1912; Poulton and Ryffel, 1913].

The present publication deals with a series of experiments performed in order to discriminate between the relative parts of nephritic retention on the one hand, and of eventual toxic overproduction of acids on the other, in the causation of the acidosis.

## *Technique.*

Dogs were kept in metabolism cages on a known diet, urine and in one case faeces and vomited matter and also intake being analysed every day for acids and bases. Blood was taken before injection of uranium nitrate (6 mg./kg.) and at the end of the experiments (6 or 7 days after intoxication).

*Chemical methods.* $p_H$ of blood by Cullen's method, with electrometric control (electrode of Vlès and Vellinger [1927]); of urine with Sb electrode of the same authors. $CO_2$ of true plasma by Van Slyke's manometric method; in urine no $CO_2$ determinations were made (the low $p_H$ excluding significant amounts). Total bases as described by Van Slyke and Peters [1932], the precipitates being collected on Jena glass filters. Cl by Rusznyak's [1921] method. Sulphate in serum gravimetrically as $BaSO_4$; in urine by the benzidine method. Inorganic phosphate in blood and urine either by Bell and Doisy's colorimetric method or micro-gravimetrically as strychnine phosphomolybdate. Protein and non-protein-nitrogen by micro-Kjeldahl. Ammonia in urine by Yovanovitch's method [1925]. The biscuit given in one experiment was analysed for N, Ca, Mg, $SO_4$ after slow dry combustion in the electric oven and for Cl and P by wet combustion.

[1] Associé du Fonds National de la Recherche Scientifique.

## Experimental.

Three dogs were used. Nos. I and II weighing 14·5 and 18 kg. received respectively every day 500 and 750 g. raw meat and distilled water *ad lib.* No vomiting occurred.

No. III received 160 g. biscuit, 15 g. fresh butter, 3 drops of lemon juice and distilled water. This food was taken regularly up to the second day after injection. A little vomiting occurred during the two following days. The next day only half of the daily intake was given and 22·6 g. dry matter were vomited. On the last day nothing was given. The daily intake contained 1·25 g. N, 36 milliequiv. Ca, 17·4 milliequiv. Mg, 3 milliequiv. Cl, traces of S and 46 milliequiv. P.

In the two first experiments urine was analysed alone, in the last one faeces were examined. The average daily faecal output before uranium was: 8·8 g. dry matter with 0·16 g. N, and, in milliequiv., 22 Ca, 6·8 Mg, 0·003 Cl and 11·2 P. After intoxication the figures were 13·8 dry matter, 0·22 g. N, and, in milliequiv., 25 Ca, 6·2 Mg, 0·004 Cl and 22 P. Together with the increase of faecal output, phosphate output goes up. As a result the excess of Ca + Mg over P drops from 17·6 to 9·1 milliequiv. which might mean an average saving of bases of 8·5 milliequiv. a day (unless S excretion changes). On the whole the faecal changes seem to be of secondary importance.

Urine analysis in milliequivalents per 24 hours for acids and bases, the average figures being given only. Separate determinations were made every day.

| No. of exp. | Period | Volume ml. | N | $NH_3$ | $NH_3$–N % | Fixed bases | Cl | P | S | Total inorganic acids | Organic acids |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I | Before uranium, 5 days | 385 | 16·5 | 34·5 | 3·2 | 36·8 | 7·4 | 49·7 | 25·0 | 82·1 | 44·3 |
| | After uranium, 7 days | 830 | 8·3 | 14·7 | 2·6 | 47·0 | 6·5 | 76·3 | 32·7 | 105·5 | 67·1 |
| | Variations, m. equiv. | +355 | − 8·2 | −19·3 | — | +10·2 | −0·9 | +26·6 | + 7·7 | +23·4 | +22·7 |
| II | Before uranium, 8 days | 514 | 26·8 | 60·0 | 3·09 | 76·0 | 13·6 | 63·0 | 44·9 | 121·5 | 63·0 |
| | After uranium, 5 days | 1015 | 13·5 | 24·0 | 3·01 | 91·0 | 14·2 | 85·4 | 39·6 | 139·2 | 64·0 |
| | Variation, m. equiv. | +501 | −13·3 | −36·0 | — | +15·0 | +0·8 | +12·4 | − 5·3 | +17·7 | + 1·0 |
| III | Before uranium, 7 days | 188 | 0·30 | 9·0 | 5·3 | 6·0 | 4·9 | 7·9 | 3·5 | 16·3 | 11·0 |
| | After uranium, 6 days | 144 | 0·14 | 3·2 | 1·2 | 9·2 | 6·2 | 5·2 | 3·2 | 14·6 | 4·8 |
| | Variation, m. equiv. | − 44 | −0·16 | − 5·8 | — | + 3·2 | +1·3 | − 2·7 | − 0·3 | − 1·7 | − 6·2 |

### *Blood analysis.*

In Exp. I the $p_H$ drops from 7·30 before to 6·98 seven days after the injection and in Exp. II from 7·40 before to 7·12 five days after. In Exp. III a full analysis of the blood is available. To give a more general idea of the alteration of the blood, we give below, together with Exp. III, the results of two other experiments.

Blood analysis (in milliequivalents).

| | Exp. III | | Exp. IV | | Exp. V | |
|---|---|---|---|---|---|---|
| | Before uranium | After uranium | Control | Nephritic | Control | Nephritic |
| Fixed base | 166·0 | 157·0 | 144·0 | 150·0 | 149·0 | 164·0 |
| Cl | 103·0 | 85·0 | 98·2 | 108·0 | 106·0 | 127·0 |
| P | 2·1 | 9·0 | 1·0 | 1·7 | 3·9 | 3·0 |
| Bicarbonate | 17·2 | 9·35 | 18·0 | 9·4 | 18·0 | 8·3 |
| S | ? | 1·45 | — | — | ? | 2·04 |
| Base bound by serum-prot. | 10·8 | 8·2 | 11·2 | 8·6 | 10·0 | 8·4 |
| Undetermined acids | 32·9 | 47·45 (without S) | 15·6 | 22·0 | 10·2 | 17·3 (2·04 S) |
| $p_H$ | 7·30 | 7·02 | 7·32 | 7·0 | 7·3 | 6·95 |

## Discussion.

The first two experiments, with high protein intake, give similar results. Ammonia output is lowered by about 50–60 %. Nevertheless the inorganic and organic acid excretions rise, probably owing to the influence of polyuria. Fixed bases behave in the same way. Although no acid retention or relative loss of bases occurs, a deep acidosis sets in after uranium nitrate.

In the third experiment, with low nitrogen intake, the ammonia excretion which is much smaller, undergoes the same decrease, with the result that its absolute output becomes very low. Oliguria sets in instead of polyuria. The inorganic acid output is not lowered, owing partly to vomiting. A relative rise of fixed bases occurs, tending to compensate the fall in ammonia. The inorganic acid output falls from 11 to 4·8 milliequiv. In this case the acidosis may be due more or less to a loss of fixed bases and perhaps to retention of organic acids.

When one considers the blood analysis, it is obvious that the rises of phosphate and sulphate are not sufficient to account for the drop of bicarbonate. The eventual rise of Cl is largely compensated by a parallel increase of bases. It seems difficult to avoid the hypothesis of an organic acid acidosis. On the other hand this hypothesis is confirmed by the results of unpublished experiments in which it was found that normal kidneys, when transferred from the circulation of a normal dog to that of a nephritic animal, show an enormous increase of organic acid output.

These findings agree with the experiments of Csapo and of Brühl referred to above.

The question, whether the acidosis is a result of toxic overproduction of acids or of a lack of catabolism owing to the general intoxication, is now being studied.

It must be said that it is very probable that the kidneys, had they retained a normal power of excretion, would have overcome by an increased excretory activity the overproduction of acids, as is shown by the crossed circulation experiments just referred to, but this would not exclude an overproduction of acids.

## Conclusions.

Acid-base balance study of experimental uranium nitrate acidosis leads to the conclusion that there is an accumulation of organic acids in the blood. This accumulation may occur independently of any retention phenomenon. It may even be found when the inorganic and organic acid output is increased, without any relative loss of fixed base.

## REFERENCES.


Brühl (1933). *Klin. Woch.* i, 72.
Brull (1931). *Comp. Rend. Soc. Biol.* **108**, 248.
—— and Fanielle (1931). *Compt. Rend. Soc. Biol.* **108**, 1163
—— —— (1932). *Arch. Internat. Pharm. Thér.* **42**, 1.
—— and Roersch (1933, 1). *Compt. Rend. Soc. Biol.* **113**, 65.
—— —— (1933, 2). *Compt. Rend. Soc. Biol.* **114**, 919.
Csapo (1932). *Z. exp. Med.* **83**, 609.
Poulton and Ryffel (1913). *J. Physiol.* **46**, *Proc.* xlvii.
Rusznyak (1921). *Biochem. Z.* **114**, 23.
Straub and Schleyer (1912). *Münch. med. Woch.* **59**, 569.
Van Slyke and Peters (1932). Quantitative clinical chemistry. Vol. II.
Vlès and Vellinger (1927). *Arch. Phys. Biol.* **6**, 38.
Weekers (1934). *Compt. Rend. Soc. Biol.* **115**, 1438.
Yovanovitch (1925). *Bull. Soc. Chim. Biol.* **7**, 665.

# CCI. ORIGIN OF VITAMIN D IN COD-LIVER OIL: VITAMIN D CONTENT OF ZOOPLANKTON.

By ALICE MARY COPPING.

*From the Division of Nutrition, The Lister Institute, London.*

(*Received June 29th, 1934.*)

THE abundance of the fat-soluble vitamins A and D in the liver of the cod has led many investigators to examine the food supplies of the cod for the source of these vitamins. In the case of vitamin A these researches have been successful. Drummond and Zilva [1922] searching for the ultimate source of vitamin A in nature found that the marine diatom, *Nitzschia closterium*, was capable of synthesising a fat-soluble growth factor. Jameson *et al.* [1922] also demonstrated that *N. closterium* could synthesise this factor under controlled conditions in the laboratory, but Leigh-Clare [1927], while confirming the synthesis of vitamin A by this organism, was unable to prove the presence of vitamin D in such cultures. Thus while the diatom might be the ultimate source of vitamin A in the food of the cod, it was extremely improbable that it could be the source of vitamin D.

As the cod is a deep-water fish, it is unlikely that any synthesis of vitamin D in the cod itself can take place by the action of ultraviolet irradiation, since such irradiation has been shown to be reduced to 1 % of its sub-surface value at a depth of 1·1–2·2 m. and to 0·001 % at 2·9–5·5 m. [Atkins and Poole, 1933]. Bills [1927] has suggested that the cod might be able to synthesise vitamin D in its liver by some process as yet undetermined, since no rich source of the vitamin has been found in its food, but Hess *et al.* [1932] were unable to show any increase of vitamin D in the livers of fish which had received ergosterol intramuscularly or by mouth. Drummond and Hilditch [1930], in examining the relative vitamin values of cod-liver oils from various sources, also investigated the food supplies of the fishing grounds and reported that there was no significant content of vitamin D in the zooplankton (copepods, *etc.*) and only very little in the small fish on which the cod was known to feed. In the same year and later, Drummond and Gunther [1930; 1934] tested extracts of zoo- and phyto-plankton, prepared as described by Collin *et al.* [1934] and showed that vitamin D was probably absent from the phytoplankton but present in the zooplankton extract, to the extent of less than 100 "Coward" (International) units per ml. (equivalence of extract in terms of original matter not stated), by giving small doses to rats in curative tests. They suggested that the zooplankton might derive the vitamin D from irradiation while in surface waters, rather than from ingestion of phytoplankton. Belloc *et al.* [1930] tested the antirachitic potency of the sterols of plankton. They found that the sterols obtained by the saponification of the oils extracted from copepods were antirachitically active without previous irradiation, but that the sterols from ctenophores required to be treated with ultraviolet light before they had any activity. The crystalline sterol from copepods was active in doses of 0·01 mg., but no figures are given by means of which this potency can be correlated with the amount of original material from which the sterol was derived.

These observations, suggesting that copepods, or the sterols extracted from them, possessed antirachitic activity, are in accord with the observation of Russell [1930] that copepods came up to the surface of the water during July, August and September, while earlier in the season they were found at depths of 10–15 m. Russell also observed that the females of the copepod *Calanus finmarchicus* were always at a slightly higher level in the water than the males during the summer.

Thus there seemed to be some evidence for the existence of vitamin D in the copepod but no quantitative data as to the amount of vitamin contained in the natural material.

### Collection and preservation of material.

Copepods are minute crustaceans which abound in the cold northern seas and form a large source of food for the young cod during the fishing season. They are not caught in the trawl, but can be collected in special fine-mesh nets. A full description of their nature and the method of collecting them is given by Gunther [1934]. Dr Atkins of the Marine Biological Station at Plymouth undertook to obtain material during the summer of 1931 and again in 1932. Numerous small catches were sent to us packed in ice.

On arrival the material was dried on trays in the hot room at 37° under an electric fan. The drying was usually complete in 24 hours, after which the material, which amounted to about 15–16 % of the original wet weight, was stored in the cold room at approximately 0° in stoppered bottles. Each batch was divided into two portions, one to be stored in the dry condition and the other after mixing with its own weight of hardened cotton-seed oil. This latter procedure was used in 1931 and the only positive result then obtained was with material stored in oil, so it was thought probable that vitamin D was more stable in oily solution. The process was therefore repeated with the 1932 material.

The catches from Plymouth consisted of mixed zooplankton from the coarse and medium mesh silk plankton nets, mainly small crustacea—copepods, with *Pseudocalanus elongatus* Boeck in largest amount, but including other common copepods as in the Plymouth Marine Fauna List.

### Experimental methods and results.

Two different methods were applied to the testing of the copepod material, but the most trustworthy results were obtained with the prophylactic method as described by Hume *et al.* [1932]. In this method young rats, at 21 days of age, were put on McCollum rachitogenic Diet 3143 [McCollum *et al.*, 1921] and given doses of the test material, or of standard vitamin D preparation or no dose (to serve as negative control) for a period of 28 days. The rats were then killed and the femur, tibia and fibula of both legs removed, dried, extracted and ashed. The degree of rickets is then calculated by the percentage of ash reckoned on the fat-extracted bone.

The line test method as modified by Coward [1928] was also used in some preliminary experiments in 1932. Young rats weaned at 21 days of age and weighing about 40 g. were kept for three weeks on McCollum Diet 3143. At the end of this period moderately severe rickets had usually developed, and doses of the materials to be tested were then given for a period of 10 days and the results assessed by examination of slices of the distal end of radius and ulna.

The copepod material received during the summer of 1931 was tested by M. M. Gaffikin and E. M. Hume, using the prophylactic method of feeding small

daily doses to young rats on a rachitogenic diet for 28 days. There was some difficulty in persuading the rats to take the copepod doses, and an extract was therefore prepared with light petroleum. Neither the extract nor the residual material was found active, but the dried copepod material preserved in cotton-seed oil proved to be a fairly good source of vitamin D in the small number of experimental animals for which it was available; 1 g. dry copepod daily produced a definite raising of the percentage ash of the bones as compared with controls receiving cotton-seed oil. The results of these tests are set out in Table I.

Table I. *Antirachitic activity of copepod material. Prophylactic experiment, 28 days on McCollum rachitogenic Diet 3143, 1931.*

| No. of rats | Supplement given daily | % ash on extracted bone (average) |
|---|---|---|
| 3 | 1 unit vitamin D (international standard) | 51·2 |
| 3 | 1 g. dried copepod in cotton-seed oil | 50·6 |
| 3 | 2 g. cotton-seed oil | 47·9 |

From May to September 1932 larger supplies of material were received and stored, the total dry weight collected being nearly 400 g. All the different samples of dry material preserved respectively alone and with cotton-seed oil, were combined into two main batches in November 1932, and some preliminary experiments using the line test curative technique were carried out. A definitely positive result was obtained by this method, 1 g. daily of either dried copepod or of copepod in cotton-seed oil when administered to rachitic rats produced rather better healing in the epiphysis than 0·25 unit of vitamin D international standard. There appeared to be no difference in antirachitic activity of the dried material whether preserved with or without cotton-seed oil.

Table II. *Antirachitic activity of copepod material. Curative experiment using rats maintained on McCollum rachitogenic Diet 3143 for line test, 1932.*

| No. of rats | Supplement given daily | Result of line test |
|---|---|---|
| 3 | 0·25 unit vitamin D (international standard) | Good healing |
| 3 | 1 g. dried copepod | Good healing |
| 3 | 1 g. dried copepod in cotton-seed oil | Good healing |
| 3 | 1 g. cotton-seed oil | No healing |
| 3 | No supplement | No healing |

The phosphorus content of the extracted copepod had been estimated as 0·8 % in 1931; the 1932 dried copepod material was found to contain about 1 % of phosphorus. It was therefore possible that the healing effects observed were due to the phosphorus administered rather than to the vitamin D in the material, since the diet used was of the high-calcium low-phosphorus type. A prophylactic experiment was therefore planned to investigate the effect, if any, of such amount of phosphorus as was given with the test material, and if necessary to allow for it.

Three litters of rats, 27 animals, at 21 days of age and weighing about 40 g. were fed on McCollum Diet 3143 without any supplementary dose for one week. At the end of this time the animals were divided, as evenly as possible, with regard to weight and sex, into four groups as follows:

(1) Rats receiving 0·125 unit of vitamin D international standard daily (6).
(2) Rats receiving 0·5 g. dried copepod daily (7).
(3) Rats receiving 5 mg. of phosphorus daily as $NaH_2PO_4$ (7).
(4) Negative control rats with no supplement (7).

The phosphorus dose was the amount calculated to be present in the dose of dried copepod and was given as a 20 % solution of sodium dihydrogen phosphate administered by means of a capillary pipette. The results are shown in Table III, which gives the percentage ash of the extracted bone and clearly indicates that the copepod dose produces good calcification.

Table III. *Antirachitic activity of copepod material. Prophylactic experiment, 1933.*

Percentage ash of extracted bones (femur, tibia and fibula) of rats on McCollum rachitogenic Diet 3143, with and without various supplements.

| Litter and sex | | Supplement given daily: 0·125 unit vitamin D | 0·5 g. dried copepod | 5 mg. phosphorus as $NaH_2PO_4$ | No dose |
|---|---|---|---|---|---|
| 2836 | ♂ | 40·90 | 49·27 | 44·40 | — |
| | ♂ | 46·22 | 51·53 | 44·36 | 30·37 |
| | ♀ | — | 52·16 | — | 32·52 |
| 2840 | ♂ | 42·63 | 48·57 | 44·92 | 41·30 |
| | ♂ | 42·93 | 53·35 | 46·94 | 30·24 |
| | ♀ | — | — | — | 35·76 |
| 2841 | ♂ | 43·57 | 50·31 | 37·60 | 36·99 |
| | ♂ | 44·18 | — | 38·02 | 41·80 |
| | ♀ | — | 52·01 | *26·00 | — |
| | Average | 43·41 | 51·03 | *40·32 | 35·56 |

* 42·70, omitting the one low figure.

## Discussion.

In spite of the recommendations of Bills *et al.* [1931] and of Dyer [1931] the line test method did not give sufficiently clear-cut results for quantitative conclusions to be drawn. In the animals used here the calcification during healing tended to be on the head of the epiphysis and not as a line in the metaphysis, so that the results were very difficult to interpret and could only be used as a very rough guide to the antirachitic potency of the doses tested. It was, however, obvious that the dried copepod material produced a marked healing effect on the rachitic animals.

In the prophylactic experiments (Tables I and III) the percentage ash of the bones of the rats which had received the copepod doses was probably a maximum figure for the ash content of bones of animals fed on a high-calcium low-phosphorus diet, and the value of the dose in the second experiment was higher than that of the standard vitamin D dose with which it was compared. As in the main experiment the rats received the supplementary doses for only three weeks out of the four-week test period the results are not strictly comparable with those obtained in the researches of Hume *et al.* [1932] and cannot be referred to the curves given in their paper. No absolute value for the antirachitic potency of the copepod material can therefore be stated. There is good agreement between figures obtained for percentage ash of the bones for any one group of rats derived from the three litters, except for one very low result on a phosphorus dose in litter 2841. This animal was difficult to dose and seemed to fare badly, and it would be reasonable to discard this result, in which case the average for the phosphorus group would be 42·70 instead of 40·45, but even so this figure is no higher than that obtained with the dose of vitamin D (0·125 unit international standard). The copepod material in the daily dose (0·5 g. dry weight) given had

therefore a much higher antirachitic potency than can be explained by its phosphorus content alone and must be regarded as a very significant source of vitamin D.

SUMMARY.

1. When tested by the line test method dried copepod material showed some indication of possessing antirachitic activity but the line test was not found satisfactory for the purpose.

2. Prophylactic experiments with a low phosphorus rachitogenic diet showed a degree of antirachitic activity in the copepod material, which was considerably greater than could be accounted for by its phosphorus content.

3. It is therefore concluded that dried copepod, while not one of the richest sources of vitamin D, yet contains sufficient of the vitamin to make this constituent of the zooplankton a good source of vitamin D in the food of the cod.

I have to thank the Medical Research Council for a part time grant which enabled this work to be done, and the Lister Institute for hospitality; also Dr Harriette Chick and Miss E. M. Hume for their helpful advice and Dr Atkins of the Marine Biological Laboratory, Plymouth, for providing the copepod material.

REFERENCES.

Atkins and Poole (1933). *Phil. Trans. Roy. Soc. Lond.* B **222**, 129.
Belloc, Fabre and Simonnet (1930). *Compt. Rend. Acad. Sci.* **191**, 160.
Bills (1927). *J. Biol. Chem.* **72**, 751.
—— Honeywell, Wirick and Nussmeier (1931). *J. Biol. Chem.* **90**, 619.
Collin, Drummond, Hilditch and Gunther (1934). *J. Exp. Biol.* **11**, 198.
Coward (1928). *Quart. J. Pharm. Pharmacol.* **1**, 27.
Drummond and Gunther (1930). *Nature*, **126**, 398.
—— and Gunther (1934). *J. Exp. Biol.* **11**, 203.
—— and Hilditch (1930). *Empire Marketing Board Rep.* No. 35.
—— and Zilva (1922). *Biochem. J.* **16**, 518.
Dyer (1931). *Quart. J. Pharm. Pharmacol.* **4**, 503.
Gunther (1934). *J. Exp. Biol.* **11**, 173.
Hess, Bills, Weinstock and Imboden (1932). *Proc. Soc. Exp. Biol. Med.* **29**, 1227.
Hume, Pickersgill and Gaffikin (1932). *Biochem. J.* **26**, 488.
Jameson, Drummond and Coward (1922). *Biochem. J.* **16**, 482.
Leigh-Clare (1927). *Biochem. J.* **21**, 368.
McCollum, Simmonds, Shipley and Park (1921). *J. Biol. Chem.* **47**, 507.
Russell (1930). *Nature*, **126**, 472.

# CCII. NARCOTICS AND BRAIN OXIDATIONS. REVERSIBILITY OF NARCOTIC ACTION *IN VITRO*.

By JUDA HIRSCH QUASTEL and ARNOLD HERBERT MAURICE WHEATLEY.

*From the Biochemical Laboratory, Cardiff City Mental Hospital.*

(*Received June 30th, 1934.*)

In earlier work the authors [1932] have shown that narcotics in general have the property of inhibiting the oxidation by brain tissue of glucose, sodium lactate and sodium pyruvate but not that of sodium succinate or of *p*-phenylenediamine. Narcotics such as luminal, chloretone or hyoscine at concentrations of 0·12 % inhibit the oxidation of glucose by brain tissue by amounts varying from 79 to 94 %. The reversibility of this inhibitory action by the narcotics was not at the time examined. This has since been done, and it is the purpose of the authors to show, in this communication, that with narcotics possessing powerful inhibitory effects, such as those already mentioned, reversibility occurs *in vitro* to the extent of at least 75 %.

*Technique.*

Minced tissue has been previously used in the work of the authors on oxidations of the brain. It had been found desirable to use brain tissue minced or chopped to as homogeneous a condition as possible in order to compare the respiration of such tissue in presence of narcotics *etc.* with that in the absence of added substances.

To test the reversibility of the inhibitory action of narcotics, it is necessary to remove the brain tissue from a medium containing a narcotic to a medium free from the narcotic, and to observe whether an increase in the respiration of the tissue takes place. With minced brain tissue this involves centrifuging and transference of the centrifuged deposit to a fresh medium. Hence it becomes important to discover whether this procedure in itself affects the subsequent respiration of the tissue when placed in a fresh medium.

The following experiment was therefore carried out: 0·5 g. minced fresh guinea-pig brain was placed in a medium containing 1 ml. *M*/5 phosphate buffer, $p_H$ 7·4, 1·5 ml. saline and 0·5 ml. of 0·5 % glucose (in saline), and the respiration followed in a Barcroft respirometer. Air in the Barcroft vessels was displaced by oxygen, the temperature at which the experiment was carried out was 37° and the manometers were allowed to shake 90 times per minute. Readings were taken every 15 minutes.

The tissue was allowed to take up oxygen for 2 hours, after which the contents of the manometer vessel were transferred to a centrifuge-tube as completely as possible, the vessel being washed out twice with 0·5 ml. of the phosphate-saline-

glucose medium. The mixture was then centrifuged for 20 minutes. By means of a pipette 3·5 ml. of centrifugate were removed and 2·5 ml. of the phosphate-saline-glucose medium were added to the centrifuged deposit. The contents of the tube were then stirred and transferred carefully to a dry manometer vessel; the air was displaced by oxygen and the rate of respiration was again measured.

The results are noted in Table I which gives the $Q_{O_2}$ as measured for each successive interval of 15 minutes. The $Q_{O_2}$ ($\mu$l. $O_2$ at N.T.P. absorbed per hour per mg. dry weight of tissue) is reckoned on the basis that the dry weight is approximately equal to one-fifth of the wet weight of the tissue. It will be seen from

Table I.

| | $Q_{O_2}$. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Time from commencement (mins.) | 15 | 30 | 45 | 60 | 75 | 90 | 105 | 120 |
| Before centrifuging | 6·7 | 6·8 | 6·3 | 6·2 | 5·7 | 5·3 | 5·2 | 5·5 |
| After centrifuging | 3·6 | 3·5 | 3·4 | 3·3 | 3·1 | — | — | — |

this table that the procedure of centrifuging, together with discarding the centrifugate, has resulted in a large drop in $Q_{O_2}$ of the minced brain tissue. The $Q_{O_2}$ had been fairly constant with the minced tissue for the hour previous to centrifuging and was also constant for the hour succeeding this operation. The drop in $Q_{O_2}$ was of the order of 40 %—a value far greater than the estimated experimental error of 5 %.

Whether the fall in $Q_{O_2}$ accompanying centrifuging and transference of the centrifuged deposit to a fresh medium is due to the removal of a factor important for the respiration of the tissue, or whether the fall is due largely to a progressive decrease of $Q_{O_2}$ with time is a matter for further investigation; so far as the present work is concerned, the conclusion is clear that the use of minced brain tissue is undesirable for demonstrating reversibility *in vitro* of narcotic action.

The question then arose as to whether brain slices could be used. From the point of view of demonstrating reversibility it is immaterial as to how far the brain slice is composed of grey or of white matter, for clearly the purpose of the experiment is to compare the $Q_{O_2}$ of a particular brain in presence of the narcotic with that after washing it as free as possible from the narcotic. It was necessary to carry out preliminary work to discover whether washing brain slices resulted in a fall in $Q_{O_2}$.

The following experiment was carried out. Small slices were cut, from the cortex as far as possible, of a fresh guinea-pig brain and placed in a medium composed of 1 ml. $M/5$ phosphate buffer solution, $p_H$ 7·4, 1·5 ml. saline, 0·5 ml. of 0·5 % glucose (in saline). This was contained in the manometer vessel of a Barcroft respirometer. Air was displaced by oxygen and the oxygen uptake of the slices at 37° was followed, readings being taken each 15 minutes. At the termination of 2 hours, the slices were removed from the manometer vessel into a dish containing a phosphate-saline-glucose medium of the composition already given which had been warmed to 37°. The slices were gently stirred in the liquid and then removed to another manometer vessel containing 3 ml. of the same medium. The oxygen uptake was again followed for an hour or 75 minutes. The slices were then carefully transferred to a dish of distilled water, stirred gently, placed on a weighed drying-dish, dried at 105° and weighed.

This experiment was repeated many times, the slices being allowed to take up oxygen for varying intervals before washing. Typical results are given in Table II.

Table II.

| Period of immersion of slices before washing (mins.) | $Q_{O_2}$. 120 | | | | 60 | | | | 30 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Time from commencement of exp. (mins.) | 30 | 60 | 90 | 120 | 15 | 30 | 45 | 60 | 15 | 30 |
| Before washing | 15·8 | 16·3 | 16·7 | 14·3 | 14·0 | 15·6 | 15·3 | 15·6 | 13·7 | 14·7 |
| After washing | 13·8 | 13·0 | — | — | 13·2 | 15·2 | 15·0 | 13·8 | 14·1 | 14·7 |

The results of Table II show that it is possible to wash brain slices without seriously affecting the magnitude of the $Q_{O_2}$. As may be expected the largest drops in $Q_{O_2}$ occur when the slices have been immersed for 2 hours before washing —there having occurred then the greatest disintegration of tissue—but the fall in $Q_{O_2}$ does not exceed 20 % of the values obtained before washing. When the slices have been immersed 30 or 60 minutes before washing, the subsequent $Q_{O_2}$ shows a fall varying from nil to 8 %.

Experience has shown that the most reproducible results occur when slices are taken which are not too thin. Slices of a thickness varying from 0·4 to 0·5 mm. are the most suitable. The slices should be trimmed, after cutting, into rectangular pieces whose areas can be readily estimated by carefully outlining them on squared paper in the dish of saline in which the slices are originally immersed. Knowing the weight of the slices and the area, the average thickness can be estimated with fair accuracy.

The preliminary results described above made it seem likely that brain slices could be used for demonstrating the reversibility of action of narcotics on oxygen uptake. The technique adopted was as follows.

Slices were cut, as far as possible from the cortex, of a fresh guinea-pig brain. About ten suitable small slices were chosen, their areas outlined and half of them were transferred to a manometer vessel containing 1 ml. $M/5$ phosphate buffer solution, $p_H$ 7·4, 1·5 ml. saline, 0·5 ml. 0·5 % glucose (in saline) and the other half to a vessel containing 1 ml. phosphate buffer solution, $p_H$ 7·4, 0·5 ml. 0·5 % glucose (in saline), 0·5 or 1 ml. of a saline solution of the narcotic under investigation together with sufficient saline to give a total volume of 3 ml. The respiration was followed in the usual way (in oxygen at 37°) for 30, 60 or 120 minutes after which the slices from each vessel were washed in a phosphate-saline-glucose medium of the composition already quoted and transferred to new manometer vessels each containing 3 ml. of the same medium. The respiration was again followed (in oxygen at 37°) for an hour or 75 minutes. The slices were then washed in distilled water, dried at 105° and weighed.

The narcotics chosen for the experiments about to be quoted were those which gave the most marked inhibitions of oxygen uptake and which belonged to different chemical types. It was felt that only by the use of such narcotics could reversibility be clearly tested. The narcotics chosen were chloretone, luminal and hyoscine, and results with these will be described in order.

*Chloretone.*

The effect of chloretone at a concentration of 0·04 % on the $Q_{O_2}$ of guinea-pig brain slices is shown in Table III *a*.

The respirations of these brain slices after washing are shown in Table III *b*.

Table III *a*.

| Time from commencement of exp. (mins.) | $Q_{O_2}$ (before washing). 15 | 30 | 45 | 60 | Average $Q_{O_2}$ | % decrease in $Q_{O_2}$ due to narcotic |
|---|---|---|---|---|---|---|
| In absence of narcotic | 14·7 | 15·6 | 15·9 | 15·9 | 15·5 | |
| In presence of 0·04 % chloretone | 4·8 | 5·1 | 4·8 | 5·3 | 5·0 | 68 |

Table III *b*.

| Time from commencement of exp. (mins.) | $Q_{O_2}$ (after washing). 15 | 30 | 45 | 60 | 75 | 90 | Av. $Q_{O_2}$ | % decrease in $Q_{O_2}$ |
|---|---|---|---|---|---|---|---|---|
| No narcotic previously administered | 14·4 | 15·3 | 14·9 | 13·8 | 13·8 | 12·9 | 14·2 | |
| 0·04 % chloretone previously added | 10·0 | 12·3 | 13·3 | 11·9 | 12·2 | 11·9 | 12·0 | 16 |

The following points are noteworthy.

1. The $Q_{O_2}$ of the brain slices throughout an experiment remains very nearly constant.

2. The effect of the narcotic is rapid, the $Q_{O_2}$ in presence of the narcotic reaching its equilibrium point within 15 minutes.

3. The $Q_{O_2}$ does not fall progressively with time as the brain slice is kept exposed to the narcotic. If the narcotic were truly toxic, a "cell-poison," such a progressive fall in respiration would be expected.

4. The effect of washing brain slices previously immersed in the narcotic solution is to raise the $Q_{O_2}$ to a level not far removed from that obtained with brain slices to which no narcotic had been added. With such washed brain slices a steady $Q_{O_2}$ is rapidly reached showing the attainment of a new equilibrium.

Tables III *a* and III *b* show that chloretone (0·04 %) reduces the $Q_{O_2}$ of guinea-pig brain slices 68 %; after washing the reduction is lowered to 16 % showing a reversibility of $\frac{52}{68} \times 100\ \% = 76\ \%$.

The effects of two different concentrations of chloretone to which brain slices were exposed for 1 hour, and the reversibilities produced by washing, are shown in Table IV. It will be observed from the results of the table that although chloretone at 0·04 % exercises a greater inhibition on $Q_{O_2}$ than chloretone at 0·02 % the reversibility is about the same in both cases.

Table IV.

| Concentration of chloretone | 0·02 % | 0·04 % |
|---|---|---|
| $Q_{O_2}$ in absence of narcotic | 15·7 | 15·5 |
| $Q_{O_2}$ in presence of narcotic | 8·1 | 5·0 |
| % decrease in $Q_{O_2}$ | 50 | 68 |
| After washing: | | |
| $Q_{O_2}$: no narcotic previously added | 14·5 | 14·2 |
| $Q_{O_2}$: chloretone previously added | 12·3 | 12·0 |
| % decrease in $Q_{O_2}$ | 15 | 16 |
| % reversibility | 70 | 76 |

*Luminal.*

Luminal was given as the sodium salt, a solution of which was brought to $p_H$ 7·4 by the addition of HCl.

The effects of luminal at a concentration of 0·08 % (as the sodium salt) on the respiration of guinea-pig brain slices and the subsequent respiration after the

brain slices had been washed are shown in Table V. In this experiment the brain slices were exposed to the action of the luminal for 2 hours before washing.

Table V.

| | $Q_{O_2}$. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Time from commencement of exp. (mins.) | 15 | 30 | 45 | 60 | 75 | 90 | 105 | 120 | Av. $Q_{O_2}$ | % decrease in $Q_{O_2}$ |
| In absence of narcotic | 14·7 | 15·8 | 16·8 | 16·2 | 15·8 | 16·7 | 14·7 | 14·3 | 15·6 | |
| In presence of luminal | 9·2 | 9·2 | 9·6 | 9·5 | 9·2 | 9·2 | 9·6 | 8·7 | 9·4 | 40 |
| After washing: | | | | | | | | | | |
| No narcotic previously added | 14·6 | 13·8 | 13·8 | 12·9 | 12·3 | — | — | — | 13·5 | — |
| Luminal previously added | 13·1 | 13·1 | 12·2 | 12·1 | 11·3 | — | — | — | 12·4 | 8 |

Reversibility $= \frac{8}{10} \times 100 = 80$ %.

As in the case of chloretone it will be noticed that a constant $Q_{O_2}$ is obtained in the presence of luminal, and after washing, the $Q_{O_2}$ is raised to a new equilibrium-point. The luminal effected a 40 % decrease in the $Q_{O_2}$ of guinea-pig brain slices which was reduced to 8 % by washing; a reversibility of 80 % was thus effected.

In an experiment where the brain slices were in contact with luminal (0·08 % as sodium salt) for only 30 minutes, complete reversibility was effected. The luminal caused a drop in $Q_{O_2}$ from 15·5 to 11·5 (33 %); after washing, the $Q_{O_2}$ of the brain slices to which no narcotic had been added was 15·4 and that of the slices to which luminal had been added was 15·5.

*Hyoscine.*

Hyoscine was added as the hydrobromide, a solution of which was brought to $p_H$ 7·4. Results with hyoscine (0·16 %) with guinea-pig brain slices are shown in Table VI. In this experiment the slices were exposed to the hyoscine for 1 hour before washing.

Table VI.

| | $Q_{O_2}$. | | | | | |
|---|---|---|---|---|---|---|
| Time from commencement of exp. (mins.) | 15 | 30 | 45 | 60 | Av. $Q_{O_2}$ | % decrease in $Q_{O_2}$ |
| In absence of narcotic | 14·0 | 15·6 | 15·3 | 15·6 | 15·1 | |
| In presence of hyoscine | 9·2 | 8·5 | 8·5 | 8·0 | 8·5 | 44 |
| After washing: | | | | | | |
| No narcotic previously added | 13·2 | 15·2 | 15·0 | 13·9 | 14·3 | |
| Hyoscine previously added | 12·6 | 14·6 | 13·2 | 13·2 | 13·4 | 6 |

Reversibility $= \frac{8}{14} \times 100 = 86$ %.

Reversibility is effected by washing brain slices previously exposed to hyoscine just as in the cases of chloretone and luminal. In the experiment quoted in Table VI the reversibility was 86 %, the hyoscine having originally produced 44 % inhibition of the rate of oxygen uptake. This is typical of many experiments.

The results of exposing brain slices to hyoscine hydrobromide (0·16 %) for varying lengths of time and the effects of washing are shown in Table VII.

Table VII.

| Duration of exposure of brain slices to hyoscine before washing (mins.) | 30 | 60 | 120 |
|---|---|---|---|
| % decrease in $Q_{O_2}$ due to the hyoscine | 44 | 44 | 49 |
| % reversibility produced by washing | 88 | 86 | 90 |

These results illustrate the fact already mentioned that the brain slices come rapidly into equilibrium with the narcotic; this occurs with chloretone and luminal as well as with hyoscine. The fact that there is no marked increase in the reduction of $Q_{O_2}$ due to hyoscine with length of exposure to the drug shows that the phenomenon under consideration (inhibition of respiration) is not due to irreversible cell poisoning, a fact confirmed by the marked increase in $Q_{O_2}$ of the brain slices after washing.

It is natural to enquire why reversibility is not in all cases of the order of 100 %. It has been shown that with luminal complete reversibility can be obtained, but with chloretone and hyoscine it has not been possible so far to exceed 90 %. It has been felt by the authors that this result is due to the incomplete washing of the slices. It is difficult to remove all traces of the narcotic by washing for a few minutes and it is conceivable that failure to arrive at reversibility greater than 90 % is due to remaining traces of the narcotic in the brain slices.

*Mescaline.*

Mescaline is a drug which gives rise to visual hallucinations. The authors [1933] have shown that it depresses the respiration of guinea-pig brain. It will now be shown that this inhibitory effect is largely reversible.

Mescaline hydrochloride (0·16 %) was added to guinea-pig brain slices in presence of the phosphate-saline-glucose medium and its effect on the $Q_{O_2}$ measured. The slices were washed after an hour's exposure to the drug and the rate of respiration of the washed slices was determined. The results are given in Table VIII.

Table VIII.

| | |
|---|---|
| Average $Q_{O_2}$ in absence of mescaline | 16·7 |
| Average $Q_{O_2}$ in presence of mescaline | 11·7 |
| % decrease in $Q_{O_2}$ = 30. | |
| After washing: | |
| Average $Q_{O_2}$: mescaline not previously added | 15·3 |
| Average $Q_{O_2}$: mescaline previously added | 14·6 |
| % decrease in $Q_{O_2}$ = 5. | |
| Reversibility = $\frac{25}{30} \times 100 = 83$ %. | |

Mescaline produced an inhibition of 30 % of the rate of oxygen uptake of guinea-pig brain slices. This inhibition was reduced by washing to 5 %, showing reversibility to the extent of 83 %.

Mescaline is 3 : 4 : 5-trimethoxy-$\beta$-phenylethylamine and it was of interest to observe whether the basic amines, whose effects on brain oxidations have already been noted [Quastel and Wheatley, 1933], behave in a reversible manner similarly to the narcotics. It was shown [1933] that many amines, including $\beta$-phenylethylamine and mescaline, and also indole resemble the narcotics in depressing the oxidation by the brain of glucose and sodium lactate and pyruvate, and in not affecting the action on sodium succinate. The inhibitory action of mescaline on brain oxidation is, as has already been shown, largely reversible.

The inhibitory action of β-phenylethylamine is also largely reversible as will be seen from the results quoted in Table IX. The brain slices in this experiment were exposed to β-phenylethylamine hydrochloride (0·16 %) for one hour.

Table IX.

| Time from commencement of exp. (mins.) | $Q_{O_2}$. 15 | 30 | 45 | 60 | Av. $Q_{O_2}$ | % decrease in $Q_{O_2}$ |
|---|---|---|---|---|---|---|
| In absence of amine | 15·2 | 15·8 | 15·5 | 15·5 | 15·5 | |
| In presence of β-phenylethylamine | 9·5 | 10·4 | 9·9 | 10·2 | 10·0 | 36 |
| After washing: | | | | | | |
| No amine previously added | 13·4 | 14·8 | 14·3 | 14·1 | 14·1 | |
| β-Phenylethylamine previously added | 12·3 | 12·4 | 2·2 | 12·4 | 12·3 | 13 |

Reversibility $= \frac{12.3}{14.1} \times 100 = 64$ %.

The reversibility is apparently not as complete as with the narcotics or as with mescaline.

On testing indole it was found that no reversibility whatever occurs. Typical results with indole are shown in Table X. In this experiment guinea-pig brain slices were exposed to indole (0·03 %) for 2 hours before washing.

Table X.

| Time from commencement of exp. (mins.) | $Q_{O_2}$. 15 | 30 | 45 | 60 | 75 | 90 | 105 | 120 | Av. $Q_{O_2}$ | % decrease in $Q_{O_2}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| In absence of indole | 16·7 | 17·1 | 17·3 | 18·3 | 17·4 | 15·8 | 16·7 | 14·9 | 16·7 | |
| In presence of indole | 11·2 | 10·9 | 10·9 | 10·6 | 10·0 | 8·2 | 6·9 | 7·5 | 9·6 | 43 |
| After washing: | | | | | | | | | | |
| No indole previously added | 14·3 | 14·0 | 14·0 | 13·5 | 12·0 | — | — | — | 13·9 | |
| Indole previously added | 7·7 | 8·3 | 8·0 | 8·3 | 6·6 | — | — | — | 8·1 | 42 |

Reversibility = nil within experimental error.

Now it was quite evident by perception of the odour alone in the vessel in the second part of the experiment that some of the indole had not been removed from the slices by washing; this would probably account for much of the lack of reversibility. It is to be noted, however, that the $Q_{O_2}$ of the brain slices in presence of the indole steadily falls so that it is possible that indole, in contrast to the substances already investigated, has some progressively toxic effect on brain tissue. It is impossible, however, to decide this until more experiments have been carried out for longer periods to determine whether the $Q_{O_2}$ drops continuously or whether an equilibrium is eventually attained. Conceivably the diffusion of indole into the cells of a brain slice is a slow process. It is significant that the $Q_{O_2}$ of the washed brain slices, which had been in indole solution and which undoubtedly still contained indole, was fairly constant.

It is possible that the simple process of washing by immersing brain slices for a few minutes in a fresh phosphate-saline-glucose medium, which is effective with such narcotics as chloretone, luminal or hyoscine, is not so effective with the basic amines. This is a matter for further investigation.

## Discussion.

The most important results arising from the work described above are as follows:

1. It is possible to wash brain slices by immersion in a phosphate-saline-glucose medium without seriously affecting the respiration of the tissue. Centrifuging minced brain tissue with transference of the centrifuged deposit to a fresh medium results in a greatly lowered respiration.

2. Brain slices rapidly come into equilibrium with narcotics such as chloretone, hyoscine and luminal, a constant $Q_{O_2}$ being established. The fact that the $Q_{O_2}$ does not fall progressively with time, even in presence of relatively high concentrations of the narcotics, shows that an irreversible destructive action on the brain cells is not taking place.

3. After washing brain slices which have been exposed to the narcotics mentioned above for intervals varying from 30 to 120 mins., the $Q_{O_2}$ rises to a new equilibrium value.

4. The reversibility is greater than 70 %, complete reversibility having been found on several occasions.

5. Reversibility of inhibitory action on respiration of brain tissue is shown also by mescaline and by $\beta$-phenylethylamine. No reversibility, however, occurs with indole, where it is evident that washing has been inadequate. It is possible, however, that indole exerts some truly toxic action which increases with time.

The proof given above that reversibility of behaviour occurs *in vitro* with the narcotics gives greater biological significance to the results obtained by the authors on the action of narcotics on oxidations by minced brain tissue. It is evident that the narcotics, at the concentration used by the authors, are not acting as cell poisons, *i.e.* in the sense of substances which bring about irreversible damage to the tissue cells. That this was the case, *i.e.* that the narcotics did not act as general cell poisons, was evident from the lack of inhibitory action of narcotics on succinate oxidation by minced brain tissue. It is likely, as has already been suggested, that the narcotics inhibit respiration by competing with a substrate (probably lactic acid) at the active surfaces of the brain cells.

Bülow [1933] considers that because she failed to obtain reversibility after exposing minced brain tissue to an acetylene-oxygen mixture for 2 hours or more, that the inhibition of glucose oxidation effected by acetylene is due to an irreversible toxic effect. This may be true in the case of acetylene but such a deduction cannot be made on the basis of her experiments alone. In the first place it is evident that acetylene does not act as a general cell poison, for, as Bülow agrees, succinate oxidation is apparently unaffected by the gas. In the second place it is possible that her failure to obtain reversibility was due to the fact that the minced brain tissue which had been exposed to acetylene for 2 hours or more was still saturated—or partially saturated—with the gas, when the acetylene atmosphere in the manometer vessels was replaced by oxygen. Her results with minced brain tissue which had been washed by centrifuging in presence of distilled water are difficult to interpret in view of the results mentioned early in this paper. It is also difficult to understand the differences which she found between the actions of acetylene and ethylene; the results of Nord [1934] should be borne in mind in considering these results. The writers have exposed brain slices to an acetylene-oxygen atmosphere and have found no marked change in $Q_{O_2}$ from that obtaining in a nitrogen-oxygen atmosphere of the same composition. It was obvious from the constancy of the $Q_{O_2}$ that the acetylene was not exercising a progressively toxic action. It is a matter for

further experiment to discover why acetylene apparently does not inhibit the respiration of fresh brain tissue (either in the form of slices or of minced material) but behaves like narcotics in causing minced brain tissue, which has been exposed to the gas for 2 hours or more, to give a diminished oxidation of glucose and not of sodium succinate.

SUMMARY.

Using brain slices it is possible to show reversibility of the strong inhibitory actions of the narcotics, chloretone, luminal and hyoscine, on brain respiration. The reversibility is demonstrated by washing the slices in a phosphate-glucose-saline medium. It occurs to the extent of at least 70 % and complete reversibility has been found in a number of instances. Reversibility has also been demonstrated with mescaline and β-phenylethylamine which also inhibit brain oxidations. No reversibility has yet been found with indole which is a powerful inhibitor of brain respiration.

Our thanks are due to the Medical Research Council for a whole time grant to one of us (A. H. M. W.) and for a grant in aid of the equipment of this laboratory.

REFERENCES.

Bülow (1933). *Biochem. J.* **27**, 1832.
Nord (1934). *Science*, **79**, 159.
Quastel and Wheatley (1932). *Proc. Roy. Soc. Lond.* B **112**, 60.
—— —— (1933). *Biochem. J.* **27**, 1609.

# CCIII. REMOVAL OF BROMINE FROM THE BLOOD IN CASES OF MENTAL DISORDER.

By JUDA HIRSCH QUASTEL
and EDMUND DENYS YATES.

*From the Biochemical Laboratory, Cardiff City Mental Hospital.*

(*Received June 30th, 1934.*)

Zondek and Bier [1932; 1933] have pointed out that in certain cases of mental disorder (particularly of the manic-depressive type) the blood-bromine is usually considerably lower than normal. Unfortunately they used a method of bromine estimation which is subject to severe criticism and hence, although their conclusions may be correct, the work requires repetition with a sound method of bromine estimation. Work in this laboratory[1], using Yates's method of bromine estimation [1933], has made it clear that the bromine of blood varies very greatly among cases of mental disorder, but that variation occurs also among normal subjects, particularly women (compare also Kuranami [1932] and Guillaumin and Merejkowsky [1933]).

The authors have made an attempt, in the work about to be described, to analyse some of the factors which may contribute to the variations in blood-bromine.

It seemed possible that the rates at which tissues remove bromine from blood may vary from patient to patient. Small amounts of bromine are normally taken in the food [Damiens and Blaignan, 1932]), but this is subject to such wide fluctuation that quantitative investigation is difficult. We have therefore given patients and normal subjects, intravenously and orally, known quantities of bromide and followed the rates at which bromine disappeared from the blood after reaching its maximum value.

### *Removal of bromine from the blood.*

A number of patients were given 1 g. of sodium bromide dissolved in 50 ml. of saline by intravenous injection. The injections were made in the morning, 2 hours after the patients had breakfasted. All patients were kept in bed during the next 5 hours. The first sample of blood (10 ml.) was taken less than 5 minutes before the injection. The bromide solution was then injected, and samples of blood were withdrawn for bromine estimation at intervals of 1, 2 and 5 hours from the time of injection. Analyses were also made 1, 4 and 7 days after the time of injection. The patients during the whole of the experimental period were on their normal diet, and received no medicinal treatment during this period.

Results with 8 patients and 3 normal cases are recorded in Table I.

The following facts are noteworthy:

(1) The calculated blood-bromine immediately after injection of the bromide, assuming even distribution throughout the blood, is of the order 15–20 mg./100 ml. The value of blood-bromine found 1 hour subsequently was of the order

[1] By Yates and Hennelly (to be published shortly).

Table I.

| Name | Diagnosis | Weight kg. | Initial blood-bromine mg./100 ml. | Blood-bromine (mg./100 ml.) Time in hours after injection | | | | | | Blood-bromine immediately following injection (calc.*) mg./100 ml. | % drop in blood-Br in 1 hr. | % drop in blood-Br in 24 hrs. | Urine (first 24 hours) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 5 | 24 | 96 | 168 | | | | Vol. (ml.) | Br mg./100 ml. | Total bromine mg. |
| Y. | Normal | 66·6 | 0·95 | 2·72 | 3·00 | 2·70 | 1·64 | 1·31 | 1·20 | 15·3 | 88·5 | 95·2 | 900 | 2·38 | 21·5 |
| F. | Normal | 65·7 | 1·46 | 2·12 | 2·45 | 2·77 | 1·76 | 1·32 | 1·28 | 15·4 | 91·5 | 98·1 | 1470 | 1·25 | 18·4 |
| P. | Normal | 56·7 | 0·83 | 3·53 | 2·95 | 3·22 | 2·61 | 1·45 | 1·22 | 17·8 | 88·0 | 90·1 | 1710 | 2·53 | 43·3 |
| J. R. | Manic-depressive | 79·7 | 0·59 | 2·48 | 2·32 | 1·87 | 2·86 | 1·40 | 0·85 | 12·7 | 86·2 | 82·4 | 1800 | 4·82 | 86·8 |
| C. E. | Dementia paranoides | 49·0 | 0·52 | 4·81 | 4·00 | 4·38 | 3·88 | — | 2·08 | 20·6 | 78·2 | 82·6 | 1050 | 3·61 | 39·7 |
| O. K. | Hebephrenia | 69·8 | 0·19 | 3·50 | 2·93 | 2·80 | 2·40 | — | 1·90 | 14·5 | 81·2 | 84·9 | 530 | 0·63 | 3·3 |
| S. | Dementia paranoides | 64·3 | 0·41 | 3·96 | 3·92 | 3·76 | 3·34 | — | 1·96 | 15·6 | 77·4 | 87·4 | 1090 | 1·64 | 17·9 |
| P. T. | Melancholia | 59·4 | 0·29 | 3·66 | 3·40 | 3·08 | 2·78 | — | 1·78 | 17·0 | 81·6 | 85·2 | 1260 | 0·42 | 5·3 |
| N. | Dementia paranoides | 53·6 | 0·14 | 3·80 | 3·48 | 3·22 | 2·78 | — | 1·83 | 18·8 | 82·2 | 86·3 | 730 | 1·19 | 8·7 |
| M. | Paraphrenia | 50·4 | 1·14 | 3·66 | 3·46 | 2·10 | 1·54 | — | 1·47 | 20·0 | 80·3 | 98·0 | 1180 | 1·08 | 12·7 |
| O. B. | Catatonia | 53·1 | 1·17 | 3·78 | 3·28 | 2·40 | 1·30 | — | 1·26 | 18·9 | 89·7 | 99·3 | 460 | 2·87 | 13·2 |

* Calculated on the basis of blood weight = 1/13 body weight.

3–4 mg./100 ml. Examination of column 10 will show that the percentage drop in blood-bromine during the one hour from the time of injection is very nearly the same in all cases examined—both normal and mentally abnormal.

It is evident that there is a rapid diffusion of bromide from the blood into the tissues, the velocity of this diffusion, at any rate in the initial stages, being, so far as we can ascertain, independent of the mental state.

(2) Although the initial blood-bromine (*i.e.* before injection of bromide) differs very greatly among the 11 cases studied, the values of blood-bromine one week after the injection of bromide bear but little relation to the initial values and are not very greatly dissimilar from each other. Examination of column 11 indicates that the percentage drop in blood-bromine 24 hours after the time of injection is much the same in all cases. This can be seen more clearly in Table II, where results with the two groups of patients (A, those with normal

Table II.

| | Av. blood-bromine (mg./100 ml.) (initial) | Av. fall % in blood-bromine | | % of total bromine in urine in 24 hrs. |
|---|---|---|---|---|
| | | 0 to 1 hr. | 1 to 24 hrs. | |
| A | 1·13 | 89·3 | 5·1 | 3·64 |
| B | 0·36 | 83·1 | 5·2 | 2·99 |

initial values of blood-bromine between 0·83 and 1·46 mg./100 ml. and B, those with low initial values) are summarised.

The maximum amount of Br in the urine after 24 hours was found to be only 86·8 mg., *i.e.* 11 % of the total Br injected.

It seems evident that the rate of removal of bromine from the blood is largely independent of the mental state, and low values observed are clearly not due to greater rates of removal from the blood into the tissues.

The variations in blood-bromine after oral dosage with sodium bromide (4 g. in 200 ml. of water) are shown in Table III.

Table III. *Blood-bromine after oral doses of sodium bromide (4 g.).*

| Name | Diagnosis | Initial value prior to bromide inges-tion | Blood-bromine (mg./100 ml.) Time in hours after oral dose | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 4 | 26 | 96 | 120 | 264 | 336 | 504 |
| E. Y. | Normal | 0·96 | 2·02 | 4·72 | — | 5·8 | 4·17 | — | — | 1·54 | — |
| C. F. | Dementia | 0·90 | — | 21·4 | 33·4 | 33·0 | — | 25·8 | — | 18·8 | — |
| G. E. | Dementia | 0·92 | — | 12·7 | 15·6 | 14·9 | 9·0 | — | 4·6 | — | 1·35 |
| S. K. | Oligophrenia | 0·81 | — | 4·71 | 3·75 | 1·35 | — | — | 0·92 | — | — |
| J. H. | Epileptic | 0·72 | — | 5·00 | 5·72 | 5·35 | — | 1·8 | — | 1·05 | — |
| S. | Manic depressive | 2·15 | — | — | 11·1 | 11·9 | — | 6·85 | — | — | — |
| P. J. | Dementia | 1·47 | — | 16·9 | — | — | — | — | — | — | — |
| W. | Melancholia | 0·67 | — | 9·8 | 9·9 | 8·95 | — | — | — | 3·85 | — |
| D. O. | Hebephrenia | 0·20 | — | 5·90 | 8·4 | 6·6 | — | 3·75 | — | — | — |
| J. R. (3 g. NaBr given) | Manic depressive | 0·62 | 5·62 | 5·22 | 5·90 | 4·96 | — | — | 3·55 | — | 1·80 |

The whole picture is much more complicated than that seen after intravenous injection—there are differences between the patients, doubtless in their rates of absorption of the relatively large dose of bromide into the blood-stream [compare Toxopéus, 1930; Zondek, 1933; Baur and Oppenheimer, 1922].

It is important to note, however, that the patients with low initial blood bromine values show a decided increase in blood-bromine, our figures lending

little support to the supposition that patients with low initial blood-bromine values owe such low values to an inability to absorb bromine into the blood-stream.

*Distribution of bromine in the tissues.*

In order to obtain some further details of the way in which bromine distributes itself in the animal organism we have made a complete analysis of a rabbit killed after intravenous treatment with 39 mg. (0·015 g./kg.) sodium bromide. The highest concentrations were found in the blood (2·85 mg./100 ml.), heart (2·80 mg./100 ml.), kidney (2·03 mg./100 ml.), spleen (1·90 mg./100 ml.) and stomach (1·40 mg./100 ml.). It is significant that, as in other investigations with higher concentrations of bromide, this halogen is not found concentrated largely in one particular organ, but is found distributed generally throughout the organism. Ellinger and Kotake [1911] consider that the subcutaneous tissues and liver are the largest bromide depots, while Toxopéus [1930] finds that about one-third of the bromide given to dogs by the mouth is associated with muscular tissue, further large amounts being found in the skin and gastro-intestinal system [*cf.* also Purjez *et al.*, 1933]. The concentration of bromide in these organs does not however differ very considerably from that in the other organs.

Experiments on human subjects have hitherto been confined to studies of the blood, cerebrospinal fluid and urine. Early investigation showed however that bromide replaces chloride in the gastric secretions [Nencki and Shoumow-Simanowsky, 1894], and indicated the possibility that the intestinal system could accumulate fairly large proportions of injected bromide. We were led therefore to investigate the distribution of bromine between blood and gastric juice in patients without previous bromide treatment.

*Bromide in the gastric juice.*

Specimens of resting gastric juice and blood were taken from a series of 12 psychotic patients following a 12-hour fast. Estimation of the bromine content gave the following results (Table IV).

Table IV.

| Patient | Blood-Br mg./100 ml. | Gastric Br mg./100 ml. | Ratio. $\frac{\text{Gastric Br}}{\text{Blood-Br}}$ |
|---|---|---|---|
| F. H. | 0·42 | 1·10 | 2·62 |
| H. R (1) | 0·54 | 1·87 | 3·45 |
| O. K. | 0·57 | 1·12 | 2·00 |
| L. T. | 0·60 | 1·73 | 2·88 |
| H. R. (2) | 0·66 | 1·46 | 2·21 |
| A. R. | 0·78 | 1·06 | 1·36 |
| A. M. | 0·90 | 0·80 | 0·89 |
| G. M. | 1·09 | 1·03 | 0·95 |
| H. B. | 1·14 | 0·24 | 0·21 |
| G. G. | 1·15 | 0·74 | 0·64 |
| J. G. | 1·26 | 0·62 | 0·49 |
| J. T.* | 9·0 | 20·0 | 2·22 |
| P. S.* | 10·1 | 20·9 | 2·05 |

* These patients had had previous bromide treatment.

The following points appear clear:

The bromine content of gastric juice is high and often exceeds that of blood.

The distribution of bromine between blood and gastric juice tends to favour blood at normal blood-bromine concentrations (0·7 to 2·0 mg./100 ml.).

When the blood-bromine falls lower than this normal level the distribution ratio rises in favour of gastric juice.

A possible explanation for the variation in the levels of blood-Br is thus suggested, the inverse relationship between the amounts of Br in blood and gastric juice indicating the possibility that a low blood-bromine level may result from a hypersecretion of bromine into the gastric juice.

In confirmation of this a study of 4 patients following a standard Rehfuss test meal (consisting of 600 ml. of thin gruel containing 0·08 mg./100 ml. Br administered after a 12-hour fast), showed that gastric bromine increases from 0·1 mg./100 ml. to values ranging from 0·78 to 1·25 mg./100 ml. Moreover the administration of such a meal to two patients showed that blood-bromine fell steadily during digestion (see Table V). A similar although very much smaller effect is observed with chloride [Peters and Van Slyke, 1931].

Table V.

| Patient A | | Patient B | |
|---|---|---|---|
| Time (mins.) after giving test meal | Blood-bromine mg./100 ml. | Time (mins.) | Blood-bromine mg./100 ml. |
| −45 | 1·01 | −5 | 0·80 |
| 0 | 1·08 | 0 | — |
| 45 | 0·99 | 50 | 0·85 |
| 80 | 0·83 | 95 | 0·76 |
| 120 | 0·95 | 125 | 0·63 |
| 150 | 0·77 | 175 | 0·59 |

These results indicate therefore that gastric secretion plays some part in controlling the level of bromine in the blood, and that the interpretation of variations in blood-bromine [*cf.* Kuranami, 1933; Ewer, 1932] must include a consideration of this fact.

## Summary.

1. The rate of removal of bromine from the blood, after intravenous injection of sodium bromide, is independent of the mental state. Variations in the initial blood-bromine levels, found both in normal and mentally abnormal cases, cannot be attributed to differences between the rates of removal of bromine from the blood into the tissues.

2. After oral ingestion of sodium bromide, the blood-bromine rises markedly in all patients, independently of the mental state. Low blood-bromine levels found in certain psychotic patients cannot therefore be ascribed to lack of absorption of bromine into the blood-stream.

3. Bromine may appear in the gastric juice in concentrations greater than that in the blood.

4. During the process of digestion blood-bromine falls and the bromine in gastric juice rises. It is suggested that secretion of bromine into the gastric juice plays some part in determining the normal level of blood-bromine.

Acknowledgment is due to the Medical Research Council for a whole-time grant to one of us (E. D. Y.). We also wish to express our indebtedness to the medical staff, and particularly to Dr Hennelly and Dr Ström-Olsen whose assistance in providing us with blood from their patients made this work possible.

REFERENCES.

Baur and Oppenheimer (1922). *Arch. exp. Path. Pharm.* **94**, 1.
Ellinger and Kotake (1911). *Arch. exp. Path. Pharm.* **65**, 87.
Ewer (1932). *Z. klin. Med.* **122**, 244.
Damiens and Blaignan (1932). *Compt. Rend. Acad. Sci.* **194**, 2077.
Guillaumin and Merejkowsky (1933). *Compt. Rend. Soc. Biol.* **93**, 1428.
Kuranami (1932). *J. Biochem.* (Japan), **15**, 205.
—— (1933). *J. Biochem.* (Japan), **18**, 417.
Nencki and Shoumow-Simanowsky (1894). *Arch. exp. Path. Pharm.* **34**, 313.
Peters and Van Slyke (1931). Quantitative clinical chemistry, **1**, p. 1036.
Purjez, Birkesey and Gönczi (1933). *Arch. exp. Path. Pharm.* **173**, 553.
Toxopéus (1930). *Arch. exp. Path. Pharm.* **149**, 263.
Yates (1933). *Biochem. J.* **27**, 1763.
Zondek (1933). *Klin. Woch.* 1411.
—— and Bier (1932). *Klin. Woch.* 633.
—— —— (1933). *Klin. Woch.* 55.

# CCIV. THIOLACETIC ACID AS A REAGENT FOR THE DETERMINATION OF THE INORGANIC IRON CONTENT OF CERTAIN BIOLOGICAL MATERIALS.

By SIDNEY LIONEL TOMPSETT.

*From the Biochemical Laboratory of the Institute of Pathology of the Royal Infirmary and University of Glasgow.*

(*Received June 30th, 1934.*)

The thiocyanate reaction for the determination of iron has been criticised by many writers.

However, if conditions are carefully controlled it gives accurate results but is not very suitable for the direct determination of iron in the inorganic form in extracts of biological material owing to the interference of many organic substances. Since certain of the analyses recorded in this paper were made by this method a discussion of the influence of various conditions is suitable at this stage. Pyrophosphates seriously interfere by depressing the reaction partly or completely. When dealing with biological material containing much phosphorus a wet ashing method in which an excess of sulphuric acid is present at the end of the reaction is essential. Elvehjem [1930] has employed a dry ashing method, after which the ash was boiled with alkali to convert pyrophosphates into orthophosphates. Such a technique appears to invite contamination due to the solvent action of hot alkali on glass. Kennedy [1927] and Elvehjem and Hart [1926] state that pyrophosphates are not formed during sulphuric-perchloric acid digestion but the present writer has noted that unless excess of sulphuric acid is present at the end of the reaction, pyrophosphates are liable to be formed. When 50 ml. of urine were digested with sulphuric-perchloric acid using only 1 ml. of sulphuric acid, no reaction with the thiocyanate reagent was obtained although iron was known to be present. When 5 ml. of sulphuric acid were used, satisfactory results were obtained. Marlow and Taylor [1934] however in determining iron in urine used 100 ml. urine and although they started with excess of sulphuric acid, namely 10 ml., digested to 0·5 ml. and claim to have obtained a reaction for iron with the thiocyanate reagent. Another important factor is the quantity of thiocyanate added. McFarlane [1932, 1] has shown that the quantity of thiocyanate added influences the depth of colour obtained very considerably. With increasing concentration of thiocyanate the depth of colour increases very rapidly, after which it remains constant. It is obvious from this work that the quantity of thiocyanate to be added should be sufficient to produce the maximum colour. Marlow and Taylor [1934] in their work on the iron content of urine failed to appreciate this and used such a concentration of thiocyanate that an error of a drop or two of the reagent would greatly influence the depth of colour.

*Thiolacetic acid.*

The colour produced when a solution of thiolacetic acid and iron is made faintly alkaline was first described by Andreasch [1879], who believed it to be due to the formation of a complex ferric thiolacetate, $Fe(S.CH_2COOH)_3$. It

was further stated that the colour faded after some time and was regenerated by shaking with air.

Lyons [1927] however has shown that the test is applicable to both ferrous and ferric salts. In the presence of alkali they give pink colours in very dilute solutions and reddish purple colours in more concentrated solutions. Lyons believes that when thiolacetic acid is added to a ferric salt in acid solution, a ferric salt, $Fe(S.CH_2COOH)_3$, is formed, which is immediately reduced to a ferrous salt, $Fe(S.CH_2COOH)_2$. This in alkaline solution gives an intensely coloured ion, $Fe(S.CH_2COO)_2^=$. The presence of oxidising agents prevents the formation of the colour probably owing to the formation of dithioglycollic acid which does not give the test. Lyons found that strong bases produced rapid colour fading and that ammonia was the most satisfactory to use. Michaelis and Barron [1929] believe that the colour in alkaline solution is not simply the ionised form of the ferrous complex which is colourless in acid solution but the oxidised form of the ferrous complex. More recently Hanzal [1933] and Burmester [1934] have applied the thiolacetic acid test to the determination of iron in biological material.

Lyons who first reported the reaction to be quantitative showed that it was uninfluenced by a large number of substances both organic and inorganic.

The present writer has attempted to apply the reaction to the direct determination of inorganic iron in biological material. In the first place the range of the reaction was studied. A standard iron solution was prepared containing 0·0855 g. of iron alum in 1000 ml. of 1 % sulphuric acid (1 ml. = 0·01 mg. Fe). Into a series of tubes were measured 0·001, 0·002, 0·003, 0·004, 0·005, 0·01, 0·02, 0·03, 0·04 and 0·05 mg. Fe. These were diluted to 5 ml. with distilled water, 6 drops thiolacetic acid added and then 0·5 ml. of ammonia sp. gr. 0·880. It was found that in the tubes containing 0·005 to 0·05 mg. Fe the colours were strong enough for comparison in a colorimeter and on comparison the depth of colour was found to be proportional to the concentration of iron.

Although distinct differences of colour were obtained in the tubes containing less than 0·005 mg. Fe yet they were too pale to compare in a colorimeter. In determining amounts of iron of this order it is best to use direct comparison with a set of tubes such as the above.

In the following work, all filter-papers used were washed with dilute HCl, distilled water and dried and the trichloroacetic acid was distilled *in vacuo*.

*The iron of the hen's egg.*

Nearly all the iron of the hen's egg is located in the yolk and many believe that it exists in the form of an organic complex with protein-like properties. This complex was first studied by Bunge [1884] who named it "haematogen." Hugounenq and Morel [1905] regarded haematogen as the prosthetic group in the vitellin molecule and as being the precursor of the haemoglobin formed by the chick during the incubation of the egg. They found that decomposition by acids breaks off a black pigment containing 2·6 % iron, which they called "haematovin."

Hill [1931] has submitted evidence to show that all the iron in egg-yolk is present as ferric iron, probably as a colloidal ferric hydroxide and that no organic complex exists. He found that when egg-yolk is suspended in an acetate buffer and $\alpha\alpha'$-dipyridyl added no colour is obtained. On the addition of sodium hydrosulphite a red colour similar to that obtained with ferrous salts was obtained. He found that the iron content of egg-yolk as determined by this simple procedure agreed with the iron content determined after ashing.

McFarlane [1932, 2] found that lecitho-vitellin prepared from egg-yolk contains a fair percentage of iron which appears to be present in very stable combination. He therefore concludes that part at least of the iron of the egg is present as an organic complex.

Additional evidence therefore appeared to be required to decide this point.

The contents of one egg were well mixed, treated with an equal volume of 20 % trichloroacetic acid, well shaken and filtered. To 5 ml. of the filtrate 6 drops of thiolacetic acid were added followed by 0·5 ml. of concentrated ammonia. No colour was obtained. A known volume of standard iron solution was added to another portion of the filtrate and the test repeated. The colour obtained was the same as that of a control, showing that none of the constituents of the filtrate interfered with the reaction.

To the contents of another egg some iron alum solution was added and the proteins precipitated with trichloroacetic acid as before. In this case also the filtrate gave a negative thiolacetic acid test. These reactions were repeated several times and found to hold in every case.

The following experiment was then carried out. The contents of one egg were treated with 5 ml. of thiolacetic acid and well mixed. Then an equal volume of 20 % trichloroacetic acid was added, well mixed and filtered. To 5 ml. of the filtrate 0·5 ml. of concentrated ammonia was added and the mixture shaken; a positive reaction for iron was obtained.

A series of experiments was then made in which the iron content of the hen's egg as determined in the protein-free filtrate directly with thiolacetic acid was compared with the total iron of the egg determined after ashing.

The contents of an egg were well mixed and diluted to 100 ml. 10 ml. of the mixture were treated with 2 ml. of thiolacetic acid, well shaken and 15 ml. of 20 % trichloroacetic acid added. The mixture was diluted to 30 ml. and filtered. 5 ml. of the filtrate were mixed with 1 ml. of concentrated ammonia. The colour so obtained was compared in a colorimeter with a standard prepared as follows. 2 ml. of a standard iron alum solution (2 ml. = 0·02 mg. Fe) were measured into a tube. 0·33 ml. of thiolacetic acid was added followed by 2·5 ml. of 20 % trichloroacetic acid and the whole diluted to 5 ml. 1 ml. of concentrated ammonia was added and the whole well shaken.

The total iron of the egg was determined as follows. 10 ml. of egg suspension were heated in a pyrex flask with 10 ml. of concentrated sulphuric acid and perchloric acid until all the organic matter was destroyed. On cooling, the mixture was diluted to 100 ml. To 10 ml. 2 ml. of 40 % potassium thiocyanate were added and the red ferric thiocyanate extracted with 5 ml. of amyl alcohol. The amyl alcohol layer was removed and compared in a colorimeter with a

Table I. *The iron content of the hen's egg expressed in mg. Fe per whole egg.*

| | A. Whole egg | | | B. Egg-yolk | |
|---|---|---|---|---|---|
| | Thiocyanate method after ashing | Direct determination on trichloroacetic acid filtrate | | Thiocyanate method after ashing | Direct determination on trichloroacetic acid filtrate |
| 1 | 0·92 | 0·90 | 8 | 1·15 | 1·07 |
| 2 | 1·26 | 1·25 | 9 | 1·28 | 1·31 |
| 3 | 1·00 | 0·96 | 10 | 1·28 | 1·22 |
| 4 | 1·27 | 1·21 | 11 | 1·30 | 1·26 |
| 5 | 1·25 | 1·17 | | | |
| 6 | 1·11 | 1·18 | | | |
| 7 | 1·27 | 1·19 | | | |

standard, which was prepared with the same amount of sulphuric and perchloric acids (the perchloric acid being boiled off) and contained 0·01 mg. Fe.

The results are shown in Table I. At the same time the recovery of iron added to egg was studied using the thiolacetic acid technique. The results are shown in Table II.

Table II. *The recovery of iron added to egg and determined by the thiolacetic acid method on trichloroacetic acid filtrates.*

| | Initial iron content of sample mg. | Iron added mg. | Total iron content of sample mg. | Iron recovered mg. |
|---|---|---|---|---|
| 1 | 0·0671 | 0·0878 | 0·153 | 0·0859 |
| 2 | 0·097 | 0·070 | 0·159 | 0·062 |
| 3 | 0·072 | 0·070 | 0·136 | 0·064 |
| 4 | 0·087 | 0·088 | 0·174 | 0·087 |
| 5 | 0·088 | 0·088 | 0·172 | 0·084 |
| 6 | 0·079 | 0·088 | 0·160 | 0·081 |

It will be seen that within the limits of experimental error, the iron contents of the egg as determined by the two methods are the same, and that iron (ferric) added to egg can be recovered quantitatively.

As a check the iron contents of the egg-yolks (Nos. 8–11 in Table I) after ashing were determined with the thiolacetic acid reagent as well as with the thiocyanate reagent. The maximum difference obtained between the two methods was 4 %. The figures obtained for the iron content of the hen's egg agree closely with those obtained by Cunningham [1931] whose average figure was 0·99 mg. Fe per egg.

*The non-haemoglobin iron of whole blood and serum.*

Many writers have determined what they describe as the "non-haemoglobin" iron content of the blood or serum. The results that have been obtained may be summarised as follows:

| | Serum or plasma mg. Fe per 100 ml. |
|---|---|
| Riecker and Winters [1930] | 1·0 |
| Locke *et al.* [1932] | 0·06–0·12 |
| Marlow and Taylor [1934] | 0·4 –0·7 |
| | Whole blood |
| McIntosh [1933] | 0·5 –1·0 |

In the above investigations the blood-serum or plasma was treated with trichloroacetic acid and the iron determined in the filtrates after ashing. Such methods however do not indicate the form in which the iron is present.

Barkan [1927; 1933] stated that inorganic iron could be determined in blood or serum by incubating with 0·4 % HCl at 37° for 24 hours. This was followed by ultrafiltration, after which the iron was determined in the ultrafiltrate with thiocyanate. He obtained values of 0·084 to 0·168 mg. Fe per 100 ml. human serum and a mean value of 1·7 mg. Fe per 100 ml. whole blood. Barkan found that none of the iron of serum was ultrafiltrable unless subjected to the above treatment.

Starkenstein and Weden [1928] state that all the inorganic iron and none of the iron in organic combination is extracted from tissue or blood by boiling with 5*N* HCl and then precipitating the proteins with trichloroacetic acid. They obtained values for whole blood of the order obtained by Barkan but higher values for serum.

The present writer has investigated the iron content of blood and serum using acid. When serum was treated with an equal volume of 20 % trichloroacetic acid and filtered, the filtrate was found to give no reaction for iron with the thiolacetic acid test. When known amounts of standard iron alum solution were added to serum and treated similarly, the filtrates were in some cases found to give negative reactions, in others only very faint reactions for iron. When whole blood was so treated, definite reactions were obtained for iron in the filtrates of the order of 0·5 mg. Fe per 100 ml., but recoveries of added iron were poor. It was decided therefore to apply the technique used for the determination of iron in eggs.

10 ml. of serum were treated with 24 drops of thiolacetic acid and well mixed. Then 10 ml. of 20 % trichloroacetic acid were added, well mixed and filtered. To 5 ml. of the filtrate, 0·5 ml. of concentrated ammonia was added and shaken. Using this technique a definite reaction for iron was obtained but was too faint to compare in a colorimeter. In these cases the colour was compared with a series of standard tubes that had been freshly prepared (0·004, 0·003 and 0·002 mg. Fe). The results obtained for the inorganic iron content of serum are shown in Table III and are of the same order as those obtained by Barkan. It

Table III. *The inorganic iron content of human serum (expressed as mg. Fe per 100 ml.).*

| | | | |
|---|---|---|---|
| 1 | 0·22 | 6 | 0·20 |
| 2 | 0·18 | 7 | 0·14 |
| 3 | 0·14 | 8 | 0·16 |
| 4 | 0·14 | 9 | 0·20 |
| 5 | 0·18 | 10 | 0·12 |

is not possible to compare these figures with figures obtained by other writers since the latter would possibly include organic iron as well. It will be seen that iron added in the form of iron alum to serum could be determined quantitatively using the above technique (see Table IV).

Table IV. *Recovery of iron (ferric) added to serum (expressed as mg. Fe).*

| | Initial | Iron added | Total iron | Iron recovered |
|---|---|---|---|---|
| 1 | 0·0035 | 0·01 | 0·0128 | 0·0093 |
| 2 | 0·004 | 0·01 | 0·0131 | 0·0091 |
| 3 | 0·005 | 0·015 | 0·0196 | 0·0146 |
| 4 | 0·004 | 0·01 | 0·0142 | 0·0102 |
| 5 | 0·005 | 0·015 | 0·0192 | 0·0142 |

In the case of whole blood the following technique was employed. 5 ml. of water were added to 5 ml. of oxalated blood followed by 18 drops of thiolacetic acid and well mixed. Then 5 ml. of 20 % trichloroacetic acid were added, mixed and filtered. To 5 ml. of filtrate 0·5 ml. of concentrated ammonia was added, well mixed, and compared in a colorimeter with a standard containing 0·015 mg. Fe similarly prepared. The results obtained for the inorganic iron content of whole blood are shown in Table V and it will be seen that they are slightly lower

Table V. *The inorganic iron content of human whole blood (expressed as mg. Fe per 100 ml.).*

| | | | |
|---|---|---|---|
| 1 | 1·14 | 5 | 0·96 |
| 2 | 1·06 | 6 | 1·08 |
| 3 | 0·93 | 7 | 1·40 |
| 4 | 1·36 | 8 | 1·56 |

than those obtained by Barkan. It appears that the corpuscles contain a very much greater amount of inorganic iron than the plasma. From results shown in Table VI it will be seen that iron added to whole blood in the form of iron alum can be determined quantitatively by this technique.

Table VI. *The recovery of iron added to whole blood* (*expressed as mg. iron*).

| | Initial | Iron added | Total iron | Iron recovered |
|---|---|---|---|---|
| 1 | 0·02 | 0·015 | 0·033 | 0·013 |
| 2 | 0·02 | 0·02 | 0·042 | 0·022 |
| 3 | 0·02 | 0·01 | 0·03 | 0·010 |
| 4 | 0·026 | 0·01 | 0·037 | 0·011 |
| 5 | 0·026 | 0·015 | 0·043 | 0·017 |
| 6 | 0·026 | 0·02 | 0·045 | 0·019 |

*The iron content of urine.*

Earlier writers have reported the presence of iron in normal human urine but more recent work has thrown doubt on this.

Lintzel [1929] and Lanyar *et al.* [1933] state that the iron content of urine does not exceed 0·01 mg. per litre. Henriques and Roland [1928] obtained values of 0·08 to 0·32 mg. *per diem* while Coons [1932] reports values of 0·06 to 0·48 mg. *per diem* in the urine of pregnant women. More recently Marlow and Taylor [1934] have obtained values of 0·03 to 0·8 mg. *per diem* in the urines of normal men.

Lanyar *et al.* found that the administration of iron salts by mouth produced no excretion of iron in the urine. Intravenous injection of iron salts into dogs, however, was followed by an excretion of iron into the urine. These writers were unable to detect iron in the urine in any condition other than nephrosis where there was gross albuminuria, and in these cases when the protein was coagulated by heat and filtered off the urine was found to be free of iron.

In the determination of small amounts of iron the greatest care must be taken to avoid contamination. Coons [1932] employed a method in which the urine was ashed and then heated with alkali to convert pyrophosphates into orthophosphates.

In the present study urines from males were used and were collected in paraffin-coated bottles. The following was the method employed.

50 ml. of urine were measured into a pyrex flask and 5 ml. of concentrated nitric acid added. The mixture was evaporated almost to dryness, and 5 ml. of concentrated sulphuric acid were added. The excess of nitric acid was boiled off and the residue then allowed to cool. 5 ml. of perchloric acid were added and the mixture heated until all the organic matter was destroyed and fumes driven off. A blank experiment was carried out at the same time. It was found that the reagents at present obtainable gave appreciable blanks when used in the above quantities. After cooling, the urine and the blank digest were each diluted to 20 ml., cooled, 4 ml. of 40 % potassium thiocyanate added and the red ferric thiocyanate extracted with 5 ml. of amyl alcohol. The amyl alcohol layers were separated and compared in a colorimeter with a standard prepared as follows. The standard containing 0·005 mg. Fe and 1 ml. of concentrated sulphuric acid was diluted to 10 ml. 2 ml. of 40 % potassium thiocyanate were added, and the ferric thiocyanate was extracted with 5 ml. of amyl alcohol.

The results are shown in Table VII. In the case of normal urines the colorimeter readings of the blanks and the urines were the same within the limits of experimental error, in fact these urines could not have contained more than 0·01 mg. Fe per litre. The urines from two cases of pernicious anaemia contained

Table VII. *The iron content of urine.*

| Case | Iron in mg. per litre |
|---|---|
| 1. Normal | <0·01 |
| 2. Normal | <0·01 |
| 3. Normal | <0·01 |
| 4. Normal | <0·01 |
| 5. Normal | <0·01 |
| 6. Normal | <0·01 |
| 7. Haemochromatosis | 0·16 |
| 8. Pernicious anaemia | 0·19 |
| 9. Pernicious anaemia | 1·28 |
| 10. Nephritis (gross albuminuria) | 1·72 |
| Nephritis (after precipitation of protein with trichloroacetic acid) | 1·46 |
| 11. Nephritis (gross albuminuria) | 2·33 |
| Nephritis (after precipitation of protein with trichloroacetic acid) | 2·00 |

appreciable amounts of iron—this however is in contradiction to the findings of Lanyar *et al.* [1933] who found no iron in the urine of such cases. One case of haemochromatosis showed an appreciable amount of iron in the urine. In two cases of nephritis in which there was gross albuminuria, the urines contained very large amounts of iron. The urines gave negative tests for blood and, examined microscopically, contained no red cells. It will be seen from the results that precipitation of the proteins with trichloroacetic acid removed only a very small percentage of the iron.

## Discussion.

It has been shown that thiolacetic acid is a suitable reagent for the direct determination of iron in biological extracts.

It appears that although all the iron in the hen's egg is in the inorganic form yet it is in such firm combination as to appear to be part of some organic molecule. Since iron (ferric) added to egg behaves in the same way, this can hardly be the case. This will explain the presence of iron in the lecitho-vitellin prepared by McFarlane [1932, 2] and his view that iron was actually a part of the lecitho-vitellin molecule.

The whole of the inorganic iron of serum appears to be in a very stable combination also. On the other hand part of the inorganic iron of the corpuscles appears to be more loosely combined.

As has been previously mentioned, Barkan [1933] has shown that none of the iron of serum is ultrafiltrable; this will explain the almost complete absence of iron from normal urine.

Although iron and copper are closely related, the latter apparently influencing the formation of haemoglobin from the former, they seem to differ very considerably in their physical properties. The present writer [1934] has shown that filtrates prepared from either whole blood or plasma by precipitation of the proteins with trichloroacetic acid contain all the copper. Fairly large amounts of copper are also excreted in the urine. Rabinowitch [1933] has found human urine to contain from a trace to 0·41 mg. per litre and the present writer has obtained similar values.

The view of Hill [1931] that the iron of the hen's egg is present as a colloidal ferric hydroxide is however untenable in view of the results of this paper.

Although copper is more or less equally distributed between the corpuscles and the plasma [Tompsett, 1934], inorganic iron appears in much greater concentration in the corpuscles.

Lintzel [1931] and Heubner [1926] state that ferric but not ferrous iron readily forms complexes with proteins. It is believed therefore that iron is absorbed from the digestive tract as ferrous iron.

The views of Lintzel and Heubner are suggestive since thiolacetic acid reduces ferric iron to the ferrous condition and also releases the iron of the egg from its complex.

Whether the inorganic iron of the corpuscles and plasma is in the ferric or ferrous condition is not at present known.

## Summary.

1. The whole of the iron of the hen's egg is in the inorganic form.
2. Normal human urine contains less than 0·01 mg. Fe per litre.
3. Human corpuscles contain about twenty times as much inorganic iron as the plasma.

In conclusion I wish to thank Dr D. P. Cuthbertson for his advice and the Medical Research Council for a grant towards expenses.

## REFERENCES.

Andreasch (1879). *Ber. deutsch. chem. Ges.* **12**, 1391.
Barkan (1927). *Z. physiol. Chem.* **171**, 179, 194.
—— (1933). *Z. physiol. Chem.* **216**, 1.
Bunge (1884). *Z. physiol. Chem.* **9**, 49.
Burmester (1934). *J. Biol. Chem.* **105**, 189.
Coons (1932). *J. Biol. Chem.* **97**, 215.
Cunningham (1931). *Biochem. J.* **25**, 1267.
Elvehjem (1930). *J. Biol. Chem.* **86**, 466.
—— and Hart (1926). *J. Biol. Chem.* **67**, 43.
Hanzal (1933). *Proc. Soc. Exp. Biol. Med.* **30**, 846.
Henriques and Roland (1928). *Biochem. Z.* **201**, 479.
Heubner (1926). *Klin. Woch.* **5**, 588.
Hill (1931). *Proc. Roy. Soc. Lond.* B **107**, 205.
Hugounenq and Morel (1905). *Compt. Rend. Acad. Sci.* **140**, 1065; **141**, 848.
Kennedy (1927). *J. Biol. Chem.* **74**, 385.
Lanyar, Lieb and Verdino (1933). *Z. physiol. Chem.* **217**, 160.
Lintzel (1929). *Z. Biol.* **89**, 89.
—— (1931). *Ergebn. Physiol.* **31**, 844.
Locke, Main and Rosbash (1932). *J. Clin. Invest.* **12**, 967.
Lyons (1927). *J. Amer. Chem. Soc.* **49**, 1919.
Marlow and Taylor (1934). *Arch. Int. Med.* **53**, 551.
McFarlane (1932, 1). *Biochem. J.* **26**, 1034.
—— (1932, 2). *Biochem. J.* **26**, 1061.
McIntosh (1933). *J. Clin. Invest.* **12**, 967.
Michaelis and Barron (1929). *J. Biol. Chem.* **83**, 191.
Rabinowitch (1933). *J. Biol. Chem.* **100**, 479.
Riecker and Winters (1930). *Amer. J. Physiol.* **92**, 196.
Starkenstein and Weden (1928). *Arch. exp. Path. Pharm.* **134**, 278.
Tompsett (1934). *Biochem. J.* **28**, 1544.

# CCV. THE COPPER CONTENT OF BLOOD.

By SIDNEY LIONEL TOMPSETT.

*From the Biochemical Laboratory of the Institute of Pathology of the Royal Infirmary and University of Glasgow.*

(*Received June 30th, 1934.*)

From experiments on suckling rats it has been shown that copper has an important influence in the formation of haemoglobin and the red blood cell. The Wisconsin workers [Hart *et al.*, 1927; 1928; Waddell *et al.*, 1929; Elvehjem and Sherman, 1932] have shown that new-born rats fed on a diet of cow's milk after weaning develop an anaemia. Addition of purified iron salts to the diet causes no abatement of the anaemia although iron is stored in the liver. The addition of copper however produces a marked reticulocyte response and haemoglobin increase. These results have been confirmed by others [Cunningham, 1931; Josephs, 1932; Muntwyler and Hanzal, 1933]. In addition Cunningham and Josephs have shown that copper feeding reduces the iron in the liver and tissues and increases that in the blood.

In view of the importance of this work it appears that a knowledge of the variations of the copper content of blood in various conditions would be of considerable value. The copper content of blood or serum as determined by various writers is shown in Table I. It appears that the copper content of the bloods of different species and of individuals within the same species must vary widely, or most of the methods are inaccurate.

Table I.

| Author | Species | Type of blood | Result mg. per 100 ml. |
|---|---|---|---|
| 1. Elvehjem *et al.* [1929] | Horse | Whole blood | 0·058 |
| 2. Schönheimer and Oshima [1929] | Human | Whole blood | 0·113–0·114 |
| 3. Abderhalden and Møller [1928] | Horse | Serum | 0·19 |
| 4. McFarlane [1932] | Human | Whole blood | 0·185–0·210 |
| ,, | Pig | ,, | 0·163–0·180 |
| ,, | Ox | ,, | 0·185–0·226 |
| 5. Locke *et al.* [1932] | Human | Serum | 0·08–0·095 |
| ,, | Rabbit | ,, | 0·05–0·072 |
| ,, | Horse | ,, | 0·09 |
| 6. Warburg and Krebs [1927] | Human | ,, | 0·124 |
| ,, | Rabbit | ,, | 0·104 |
| ,, | Pig | ,, | 0·194 |
| 7. McHargue [1925] | Ox | Whole blood | 0·14 |
| 8. Guillemet [1931] | Cow | Serum | 0·058–0·082 |
| ,, | Pig | ,, | 0·067–0·118 |
| ,, | Human | Plasma | 0·056–0·075 |

Guillemet and Schell [1931] used a method involving the dry ashing of blood, precipitation of copper as hydroxide, followed by filtration, dissolution in acid and deposition on platinum by electrolysis. The copper was then dissolved in "reactive phosphomolybdic acid" to form a blue solution and the copper

determined by titration with standard permanganate solution until the blue colour disappeared. These writers determined amounts of copper of the order of 0·1 mg. by this method. The number of precipitations renders this method unsatisfactory and probably accounts for the low values obtained by this method.

Warburg [1927] has devised an ingenious method for the determination of copper in serum utilising the fact that copper catalyses the oxidation of cysteine to cystine. To prevent the interference of iron, sodium pyrophosphate is added. Warburg found that copper could be determined directly in serum without the necessity of ashing.

Some writers analysing large samples of blood have used macro-chemical methods. Among those may be mentioned McHargue [1925] and Abderhalden and Møller [1928].

Among the colorimetric methods used for the determination of the copper content of biological material are:

1. The potassium ethyl xanthate method.
2. The Biazzo method [1926] as modified by Elvehjem and Lindow [1929].
3. The diethyldithiocarbamate reaction of Callan and Henderson [1929] as modified by McFarlane [1932].

Recently the accuracy of the Biazzo method has been questioned [*cf.* Ansbacher *et al.*, 1931; Walker, 1930; Drabkin and Waggoner, 1930]. A serious disadvantage of the reaction is the interference by iron, which involves the separation of copper as sulphide at a carefully adjusted $p_H$.

In the third reaction an aqueous solution of sodium diethyldithiocarbamate is added to a solution of copper to produce a golden brown colour. Callan and Henderson [1929], who discovered the reaction, obtained satisfactory results when it was applied to the determination of copper in inorganic mixtures. McFarlane [1932] found that the brown colour could be extracted with amyl alcohol thereby increasing the sensitivity of the reaction. At the present time this is the most sensitive reaction for copper—it being possible to detect one part of copper in ten million parts of solution—and is also independent of $p_H$. Iron interferes by producing a dirty brown colour but this may be prevented by the addition of sodium pyrophosphate and carrying out the reaction in a slightly alkaline medium.

As a preliminary a slight modification of McFarlane's [1932] method for the determination of copper in blood was used. This was carried out as follows:

5 ml. of blood were measured into an 8 × 1 inch pyrex tube followed by 5 ml. of concentrated nitric acid. This was allowed to digest in a boiling water-bath until the contents of the tube were almost dry. Then the remaining organic matter was destroyed by heating with 1 ml. of concentrated sulphuric acid and perchloric acid (about 3 ml.). The contents of the tube were diluted to 10 ml. with distilled water and 5 ml. of 4 % sodium pyrophosphate solution added. The solution was treated with ammonia until slightly alkaline to litmus and then placed in a water-bath at 80° for 15 minutes. After cooling 5 ml. of amyl alcohol were added followed by 0·5 ml. of 2 % aqueous sodium diethyldithiocarbamate, and the whole was well shaken. A standard containing 0·01 mg. of copper was prepared in exactly the same way. After separating, the amyl alcohol layers were removed and filtered through filter-paper to remove suspended water and compared in a colorimeter.

Any glass apparatus that was used was cleaned first with nitric acid and then with glass-distilled water and chemicals of A.R. standard were used. Filter-papers were washed with dilute acid and distilled water and dried. The standard solution contained 0·01 mg. copper per ml. This was prepared by diluting a stock solution containing 0·3928 g. $CuSO_4, 5H_2O$ in 1 litre (1 ml = 0·1 mg. Cu). This solution contained a little sulphuric acid to prevent formation of insoluble basic copper sulphate.

Satisfactory results were obtained by this method of the order obtained by McFarlane [1932].

Locke *et al.* [1932] attempted to determine the copper content of human serum by applying the diethyldithiocarbamate reagent directly to trichloroacetic acid filtrates. They obtained values of 0·08–0·095 mg. per 100 ml. serum, *i.e.* about half of those obtained by McFarlane and the present writer for whole blood (see Table III, first column). The possibility of estimating copper in protein-free filtrates without the necessity of ashing was therefore studied.

As a preliminary various substances, many of which are normally present in protein-free extracts of blood or sera, were added to a series of tubes each containing 0·01 mg. Cu. The amounts of these substances added are shown in Table II. To each of these tubes were added 2 ml.

Table II.

| Substance | Amount used mg. | Substance | Amount used mg. |
|---|---|---|---|
| Glucose | 5 | Thiolacetic acid | 5 |
| Tyrosine | 1 | Urea | 5 |
| Alanine | 5 | Glycine | 5 |
| Glutathione | 5 | Potassium cyanide | 10 |
| Glutathione | 10 | | |

of 4 % sodium pyrophosphate and then ammonia until faintly alkaline to litmus. 5 ml. of amyl alcohol were added to each followed by 0·5 ml. of 2 % sodium diethyldithiocarbamate solution and the mixture well shaken. The only substance found to prevent the reaction was potassium cyanide. In all the other cases the same colour was produced as in the control. In the cases of thiolacetic acid and glutathione, on the addition of the reagent no colour was produced until the contents of the tubes were vigorously shaken when the amyl alcohol layers were found to match the control. This was probably due to the reduction of the copper to the cuprous condition by the free SH groups, this being reversed on shaking with air.

Trichloroacetic acid filtrates were prepared as follows. The trichloroacetic acid was distilled *in vacuo* and then made up to 20 % with distilled water.

1 volume of blood, plasma or serum was diluted with 3 volumes of distilled water and then 1 volume of 20 % trichloroacetic acid added. After shaking the mixture was filtered. To 20–25 ml. of filtrate were added 2 ml. of 4 % sodium pyrophosphate and the whole made faintly alkaline to litmus with ammonia. 5 ml. of amyl alcohol were then added followed by 0·5 ml. of diethyldithiocarbamate reagent and the whole vigorously shaken. A standard containing 0·01 mg. copper was similarly prepared.

Results obtained by this method were compared with those obtained after destruction of the organic matter by the method described earlier in this paper and are shown in Table III.

It will be seen that the whole of the copper is present in the trichloroacetic acid filtrate in such a form as to react directly with the reagent.

Table III. *Copper in mg. per 100 ml. whole blood or serum.*

| | | A. Complete destruction of organic matter | B. Direct determination on trichloroacetic acid filtrate |
|---|---|---|---|
| Whole blood: | 1 | 0·174 | 0·186 |
| | 2 | 0·169 | 0·174 |
| | 3 | 0·157 | 0·171 |
| | 4 | 0·165 | 0·156 |
| | 5 | 0·200 | 0·192 |
| | 6 | 0·192 | 0·199 |
| Serum: | 7 | 0·256 | 0·263 |
| | 8 | 0·204 | 0·205 |
| | 9 | 0·238 | 0·235 |
| | 10 | 0·220 | 0·229 |
| | 11 | 0·196 | 0·204 |

Further results shown in Table IV indicate that copper added to whole blood or serum could be recovered quantitatively.

Table IV. *Recovery of copper added to whole blood or serum.*

| | Copper content of blood *etc.* mg. Cu per 100 ml. | Copper added mg. Cu per 100 ml. | Total copper determined mg. Cu per 100 ml. | Copper recovered mg. Cu per 100 ml. |
|---|---|---|---|---|
| Whole blood: | | | | |
| 1 | 0·156 | 0·143 | 0·289 | 0·133 |
| 2 | 0·156 | 0·286 | 0·435 | 0·279 |
| 3 | 0·156 | 0·429 | 0·683 | 0·427 |
| 4 | 0·223 | 0·143 | 0·378 | 0·155 |
| 5 | 0·223 | 0·286 | 0·500 | 0·277 |
| Serum: | | | | |
| 1 | 0·245 | 0·143 | 0·378 | 0·133 |
| 2 | 0·245 | 0·286 | 0·526 | 0·281 |
| 3 | 0·217 | 0·143 | 0·351 | 0·134 |
| 4 | 0·217 | 0·143 | 0·356 | 0·139 |
| 5 | 0·217 | 0·286 | 0·490 | 0·273 |

The question naturally arises as to the distribution of copper between the corpuscles and plasma. Guillemet [1932] found much higher concentrations of copper in the plasma than in the whole blood of the ox, pig and dog. Schindel [1931] obtained higher values for the copper content of the plasma than the cells in man. Elvehjem *et al.* [1929] however obtained more copper in the corpuscles than the plasma in defibrinated horse blood. McHargue *et al.* [1928] in the case of one cow found the plasma to contain more copper than the corpuscles. This animal does not appear to have been normal. The values obtained by Elvehjem *et al.* and Guillemet are very much lower than those obtained by the writer, Schindel's figures however are of the same order.

For the study of the distribution of copper in the blood of man, serum from clotted blood and plasma from citrated blood were used. It was considered that the use of oxalate to prevent clotting might possibly cause a loss of copper from the plasma.

Table V. *Distribution of copper between plasma and corpuscles of human blood.*

| | Whole blood mg. Cu per 100 ml. | Serum mg. Cu per 100 ml. | | Whole blood mg. Cu per 100 ml. | Plasma from citrated blood mg. Cu per 100 ml. |
|---|---|---|---|---|---|
| 1 | 0·184 | 0·197 | 9 | 0·225 | 0·238 |
| 2 | 0·225 | 0·245 | 10 | 0·227 | 0·227 |
| 3 | 0·174 | 0·183 | 11 | 0·208 | 0·227 |
| 4 | 0·192 | 0·209 | 12 | 0·238 | 0·227 |
| 5 | 0·195 | 0·197 | 13 | 0·220 | 0·236 |
| 6 | 0·210 | 0·217 | 14 | 0·207 | 0·220 |
| 7 | 0·238 | 0·205 | | | |
| 8 | 0·202 | 0·220 | | | |

From the results shown in Table V it will be seen that copper is more or less equally distributed between the cells and the plasma. In Table VI are shown the copper contents of the bloods of 22 normal males and 10 normal females. It will be seen that no apparent difference exists between the sexes—the values ranging from 0·185 to 0·229 mg. per 100 ml.

Four samples of cerebrospinal fluid were examined for copper. The copper content ranged from 0·032 to 0·067 mg. per 100 ml.

Table VI. *The copper content of normal human blood (expressed in mg. per 100 ml. blood).*

| Male | | | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0·187 | 9 | 0·198 | 16 | 0·194 | 1 | 0·208 | 6 | 0·198 |
| 2 | 0·197 | 10 | 0·222 | 17 | 0·186 | 2 | 0·228 | 7 | 0·196 |
| 3 | 0·220 | 11 | 0·189 | 18 | 0·218 | 3 | 0·198 | 8 | 0·214 |
| 4 | 0·229 | 12 | 0·219 | 19 | 0·208 | 4 | 0·216 | 9 | 0·196 |
| 5 | 0·220 | 13 | 0·194 | 20 | 0·194 | 5 | 0·196 | 10 | 0·216 |
| 6 | 0·202 | 14 | 0·192 | 21 | 0·216 | | | | |
| 7 | 0·185 | 15 | 0·207 | 21 | 0·189 | | | | |
| 8 | 0·194 | | | | | | | | |

Owing to the widely different values reported for the copper contents of the blood and plasma (or serum) of different species, analyses of the whole blood and plasma (from citrated blood) from various animals were made. From the results shown in Table VII it will be seen that the values are of the same order as for the human being in the case of the ox, sheep, pig, horse and guinea-pig, while in the case of the rabbit they are slightly lower. McFarlane [1932], who analysed the blood of the ox and of the pig, obtained similar results to those of the writer.

Table VII. *The copper content of the whole blood and plasma of various species expressed in mg. per 100 ml.*

| | | Whole blood | Plasma |
|---|---|---|---|
| Sheep: | 1 | 0·163 | 0·172 |
| | 2 | 0·167 | 0·156 |
| | 3 | 0·172 | 0·172 |
| | 4 | 0·180 | 0·183 |
| | 5 | 0·156 | 0·161 |
| Ox: | 1 | 0·223 | 0·208 |
| | 2 | 0·200 | 0·208 |
| | 3 | 0·190 | 0·180 |
| | 4 | 0·180 | 0·190 |
| | 5 | 0·192 | 0·192 |
| Pig: | 1 | 0·181 | 0·191 |
| | 2 | 0·165 | 0·161 |
| | 3 | 0·168 | — |
| | 4 | 0·185 | 0·200 |
| | 5 | 0·167 | 0·185 |
| Horse: | 1 | 0·179 | 0·167 |
| | 2 | 0·187 | 0·199 |
| | 3 | 0·208 | 0·208 |
| | 4 | 0·205 | 0·217 |
| Rabbit: | 1 | 0·143 | — |
| | 2 | 0·147 | — |
| | 3 | 0·144 | — |
| | 4 | 0·155 | — |
| | 5 | 0·139 | — |
| Guinea-pig: | 1 | 0·192 | — |
| | 2 | 0·186 | — |
| | 3 | 0·179 | — |
| | 4 | 0·184 | — |

## Discussion.

It has been shown that the diethyldithiocarbamate reagent offers a simple and reliable method for the determination of copper in blood. Since the whole of the copper is present in the filtrate after the precipitation of the proteins with trichloroacetic acid and is in such form that it may be determined directly—ashing is not necessary. It does not appear from these findings that any copper

complex analogous to haemoglobin exists in the blood. This is to be expected since even the haemocyanins are unstable. Roche [1930] has shown that it is possible to separate copper from haemocyanin by adjusting the solution to $p_H$ 2·5 and then dialysing. Pirie [1931, 2], in a study of the catalytic oxidation of sulphydryl compounds by iron and copper, found that although the haemocyanins were themselves catalytically inactive, they were decomposed at $p_H$ 2·1, yielding catalytically active copper.

Pirie [1931, 1] has shown that the addition of copper sulphate to sulphydryl compounds results in the formation of cuprous derivatives. It is to be expected therefore that part at least of the copper present inside the red blood cell will be in the cuprous condition owing to the presence of glutathione. This is also suggested by the fact that the full colour is not obtained with whole blood filtrates unless they are vigorously shaken. Owing to the absence of free SH groups from plasma the copper is present in the cupric form.

SUMMARY.

1. Normal human blood contains from 0·185 to 0·229 mg. Cu per 100 ml. which is more or less equally distributed between plasma and corpuscles.

2. The copper contents of sheep, ox, pig, horse and guinea-pig bloods are of the same order as that of human blood. Rabbit blood however shows slightly lower values.

In conclusion I wish to thank Dr D. P. Cuthbertson for his criticism and advice and the Medical Research Council for a grant towards expenses.

REFERENCES.

Abderhalden and Møller (1928). *Z. physiol. Chem.* **176**, 95.
Ansbacher, Remington and Culp (1931). *J. Ind. Eng. Chem.* **3**, 314.
Biazzo (1926). *Ann. Chim. Appl.* **16**, 2.
Callan and Henderson (1929). *Analyst*, **54**, 650.
Cunningham (1931). *Biochem. J.* **25**, 1267.
Drabkin and Waggoner (1930). *J. Biol. Chem.* **89**, 51.
Elvehjem and Lindow (1929). *J. Biol. Chem.* **81**, 435.
—— Steenbock and Hart (1929). *J. Biol. Chem.* **83**, 21.
—— and Sherman (1932). *J. Biol. Chem.* **98**, 309.
Guillemet (1931). *Compt. Rend. Soc. Biol.* **108**, 32.
—— (1932). *Compt. Rend. Soc. Biol.* **109**, 652.
—— and Schell (1931). *Compt. Rend. Soc. Biol.* **107**, 1006.
Hart, Elvehjem, Waddell and Herrin (1927). *J. Biol. Chem.* **72**, 299.
—— Steenbock, Waddell and Elvehjem (1928). *J. Biol. Chem.* **77**, 797.
Josephs (1932). *J. Biol. Chem.* **96**, 559.
Locke, Main and Rosbash (1932). *J. Clin. Invest.* **11**, 527.
McFarlane (1932). *Biochem. J.* **26**, 1022.
McHargue (1925). *Amer. J. Physiol.* **72**, 582.
—— Healy and Hill (1928). *J. Biol. Chem.* **78**, 637.
Muntwyler and Hanzal (1933). *Proc. Soc. Exp. Biol. Med.* **30**, 845.
Pirie (1931, 1). *Biochem. J.* **25**, 614.
—— (1931, 2). *Biochem. J.* **25**, 1565.
Roche (1930). *Arch. Physique Biol.* **7**, 207.
Schindel (1931). *Klin. Woch.* **10**, 743.
Schönheimer and Oshima (1929). *Z. physiol. Chem.* **180**, 249.
Waddell, Steenbock and Hart (1929). *J. Biol. Chem.* **83**, 243; **84**, 115.
Walker (1930). *J. Assoc. Offic. Agric. Chemists*, **13**, 246.
Warburg (1927). *Biochem. Z.* **187**, 255.
—— and Krebs (1927). *Biochem. Z.* **190**, 143.

# CCVI. STUDIES ON REVERSIBLE DEHYDROGENASE SYSTEMS.

## II. THE REVERSIBILITY OF THE XANTHINE OXIDASE SYSTEM.

By DAVID EZRA GREEN.

*From the Biochemical Laboratory, Cambridge.*

(*Received July 3rd, 1934.*)

Dixon [1926] attempted to measure the potential of the xanthine oxidase system (hypoxanthine in presence of the enzyme) by means of the Clark series of indicators and found that all the indicators were reduced, including the most negative, *viz.* indigodisulphonate, which has an $E_0'$ of $-0{\cdot}125$ v. at $p_H$ 7·0. The lack of reversible indicators covering more negative ranges of potential made it impossible to continue this study. In recent years, Michaelis [1931], Michaelis and Eagle [1930] and Michaelis and Hill [1933] have discovered several indicators of very negative potential, in particular rosinduline and the viologens, which cover the range of potential between $r_H$ 0 and 5. The availability of these new indicators offered the possibility of pursuing the work of Dixon, and evidence will be presented in this report to show that the systems hypoxanthine-xanthine and xanthine-uric acid are reversible.

Table I contains a list of indicators which are reduced by the xanthine oxidase from milk in the presence of hypoxanthine and xanthine respectively at $p_H$ 7·0. The results show clearly that the oxidase system lies in the range of methyl- and benzyl-viologens, and near the level of the hydrogen electrode. It is noteworthy that hypoxanthine and xanthine have comparable reducing powers.

Table I.

| Indicator | $E_0'$ at $p_H$ 7·0 and 30° | $p_H$ 7·0, 30° | |
|---|---|---|---|
| | | Hypoxanthine | Xanthine |
| Methylene blue | +0·011 | Complete reduction | Complete reduction |
| Nile blue | −0·142 | ,, | ,, |
| Cresyl violet | −0·167 | ,, | ,, |
| Phenosafranine | −0·242 | ,, | ,, |
| Janus green (rose to colourless) | −0·258 | ,, | ,, |
| Rosinduline | −0·281 | ,, | ,, |
| Benzylviologen | −0·359 | Partial reduction | Partial reduction |
| Methylviologen | −0·446 | ,, | ,, |

The reversibility of the systems hypoxanthine-uric acid and xanthine-uric acid was then demonstrated in the following qualitative fashion. Xanthine oxidase (whey preparation), hypoxanthine and methylviologen were all dissolved in phosphate buffer $p_H$ 7·4 and mixed in a Thunberg tube, and crystals of

uric acid were placed in the side-bulb of the stopper. Within a few minutes after evacuation of the tube a deep blue colour developed, representing approximately 70 % reduction of the methylviologen. The colour persisted unchanged for an hour. The crystals of uric acid were then tipped into the solution. The blue colour gradually faded until about 30 % of the total amount of the indicator remained in the reduced form. No further change occurred even after several hours. The same type of experiment was carried out replacing hypoxanthine with xanthine. Reduction in this case proceeded to only 15 % of completion. Tipping-in of uric acid resulted in the almost complete reoxidation of the indicator. Hardly a trace of blue colour was evident even after several hours. When crystals of xanthine were tipped into a solution containing hypoxanthine, oxidase and partially reduced methylviologen, the change was from 70 to 50 % reduction in contrast to the change from 70 to 30 % in the case of uric acid.

From these results the conclusion can be drawn that methylviologen comes into equilibrium with the systems hypoxanthine-uric acid, hypoxanthine-xanthine and xanthine-uric acid respectively. By alteration of the ratio of oxidant to reductant the potentials of these three systems vary, as shown by the changes in the extent of reduction of the indicator.

Whilst these studies were in progress, Filitti [1933; 1934] published two short notes dealing with the potentials of the xanthine oxidase system. She assigned $E_0' - 0{\cdot}410$ v. to the system hypoxanthine-uric acid at $p_H$ 7·24 and developed the following electrode formula:

$$E_h = E_0' - \frac{RT}{4F} \ln \frac{[HX]}{[Uric]}.$$

No evidence was presented in her notes that the observed potentials agreed with those predicted by the assigned formula. In all the various measurements the ratio of oxidant to reductant was kept constant. From her experimental data therefore she could draw no conclusions concerning the thermodynamic reversibility of the system. Furthermore, as justification for assigning the value of 4 for the number of equivalents $n$ in the electrode formula, Filitti states that both hypoxanthine and xanthine can reduce all the indicators down to and including methylviologen, and that therefore hypoxanthine and xanthine have identical reducing powers. This conclusion of course does not follow. If the equilibrium points of the systems hypoxanthine-xanthine and xanthine-uric acid are close together, the difference in reducing power will show up only with methylviologen or benzylviologen and not with any of the other oxidation-reduction indicators. Filitti has overlooked what is the greatest complication in the study of the potentials of the xanthine oxidase system. If the assumption is made that the oxidation of hypoxanthine to uric acid involves one step of four equivalents, a straightforward analysis of the system can be made with the ordinary electrode equations. But if the oxidation of hypoxanthine to uric acid occurs in two distinct steps, then a unique case develops which conforms to a special type of electrode equation.

Hitherto two-step oxidations each involving two equivalents have been rarely encountered[1] and no adequate theory has been developed to take account of such cases. It is proposed therefore to consider in some detail how two-step oxidations of the type described can be handled theoretically.

[1] Cannan [1926] encountered a two-step oxidation of two equivalents per step in his study of the potentials of hermidin. But the two steps did not overlap sufficiently to interfere with the ordinary formula.

*Theory of two-step oxidation.*

I. Suppose we start with substances A and C in certain concentrations and assume that the oxidation-reduction systems $A \rightleftharpoons B+2e$, $B \rightleftharpoons C+2e$ come into equilibrium with one another, how will the potentials depend upon the original ratio of A to C?

Originally let $[A]=a$; $[C]=c$.

At equilibrium $[A]=a-x$; $[C]=c-x$; $[B]=2x$; the equilibrium reaction being $A+C \rightleftharpoons 2B$, where $x$ represents the amount of A or of C which has disappeared, and therefore half the amount of B which has been formed by the interaction of A and C. The basic assumption in this proof is that regardless of which pair of substances is taken—A and B, A and C, or B and C—a dismutation, or internal oxido-reduction, occurs as a result of which the third substance is formed to an extent determined by the equilibrium constant for the reaction.

Also at equilibrium

$$E_h = E_{0_1}' - \frac{RT}{2F}\ln\frac{[A]}{[B]} = E_{0_2}' - \frac{RT}{2F}\ln\frac{[B]}{[C]},$$

or

$$\ln\frac{[A]}{[B]} = -\frac{(E_h - E_{0_1}')\,2F}{RT};\quad \ln\frac{[B]}{[C]} = -\frac{(E_h - E_{0_2}')\,2F}{RT}.$$

Therefore

$$\frac{[A]}{[B]} = e^{-\Delta E_1 \alpha} \text{ and } \frac{[B]}{[C]} = e^{-\Delta E_2 \alpha},$$

where $\Delta E = E_h - E_0'$ and $\alpha = \frac{2F}{RT}$.

By substitution

$$\frac{a-x}{2x} = e^{-\Delta E_1 \alpha} \text{ and } \frac{2x}{c-x} = e^{-\Delta E_2 \alpha},$$

whence

$$x = \frac{a}{1+2e^{-\Delta E_1 \alpha}} = \frac{c}{1+2e^{\Delta E_2 \alpha}},$$

and finally

$$\frac{a}{c} = \frac{1+2e^{-\Delta E_1 \alpha}}{1+2e^{\Delta E_2 \alpha}}.$$

II. Suppose we start with a mixture of B and C, how will the potentials depend upon the original ratio of concentrations?

Originally let $[B]=b$; $[C]=c$.

At equilibrium $[B]=b-2x$; $[C]=c+x$; $[A]=x$; the equilibrium reaction being $A+C \rightleftharpoons 2B$, where $x$ represents the amount of A or of C which has been formed, and therefore half the amount of B which has disappeared.

Also as before

$$E_h = E_{0_1}' - \frac{RT}{2F}\ln\frac{[A]}{[B]} = E_{0_2}' - \frac{RT}{2F}\ln\frac{[B]}{[C]}.$$

Therefore

$$\frac{x}{b-2x} = e^{-\Delta E_1 \alpha};\quad \frac{b-2x}{c+x} = e^{-\Delta E_2 \alpha},$$

and

$$x = \frac{be^{-\Delta E_1 \alpha}}{1+2e^{-\Delta E_1 \alpha}}.$$

By substitution

$$\frac{b - \dfrac{2be^{-\Delta E_1 \alpha}}{1+2e^{-\Delta E_1 \alpha}}}{c + \dfrac{be^{-\Delta E_1 \alpha}}{1+2e^{-\Delta E_1 \alpha}}} = e^{-\Delta E_2 \alpha},$$

$$b-\frac{2be^{-\Delta E_1\alpha}}{1+2e^{-\Delta E_1\alpha}}=ce^{-\Delta E_2\alpha}+\frac{be^{-(\Delta E_1+\Delta E_2)\alpha}}{1+2e^{-\Delta E_1\alpha}},$$

$$b\left[\frac{1-e^{-(\Delta E_1+\Delta E_2)\alpha}}{1+2e^{-\Delta E_1\alpha}}\right]=ce^{-\Delta E_2\alpha}.$$

Finally

$$\frac{b}{c}=\frac{e^{-\Delta E_2\alpha}+2e^{-(\Delta E_1+\Delta E_2)\alpha}}{1-e^{-(\Delta E_1+\Delta E_2)\alpha}},$$

$$\frac{b}{c}=\frac{1+2e^{-\Delta E_1\alpha}}{e^{\Delta E_2\alpha}-e^{-\Delta E_1\alpha}}.$$

III. If the starting pair of substances are A and B, the equation is derived as follows:

Originally let $[A]=a$; $[B]=b$.

At equilibrium $[A]=a+x$; $[B]=b-2x$; $[C]=x$; the equilibrium reaction being $A+C \rightleftharpoons 2B$, where $x$ has the same meaning as in case II.

Therefore

$$\frac{a+x}{b-2x}=e^{-\Delta E_1\alpha};\ \frac{b-2x}{x}=e^{-\Delta E_2\alpha},$$

and

$$x=\frac{b}{e^{-\Delta E_2\alpha}+2}.$$

By substitution

$$\frac{a+\dfrac{b}{e^{-\Delta E_2\alpha}+2}}{b-\dfrac{2b}{e^{-\Delta E_2\alpha}+2}}=e^{-\Delta E_1\alpha},$$

$$\frac{\dfrac{a\,(e^{-\Delta E_2\alpha}+2)+b}{e^{-\Delta E_2\alpha}+2}}{\dfrac{b\,(e^{-\Delta E_2\alpha}+2)-2b}{e^{-\Delta E_2\alpha}+2}}=e^{-\Delta E_1\alpha},$$

$$a\,(e^{-\Delta E_2\alpha}+2)+b=b\,(e^{-\Delta E_1\alpha})\,(e^{-\Delta E_1\alpha}).$$

Finally

$$\frac{a}{b}=\frac{e^{-(\Delta E_1+\Delta E_2)\alpha}-1}{e^{-\Delta E_2\alpha}+2},$$

$$\frac{a}{b}=\frac{e^{-\Delta E_1\alpha}-e^{\Delta E_2\alpha}}{1+2e^{\Delta E_2\alpha}}.$$

The final equations for $a/b$, $b/c$ and $a/c$ have been worked out for the cases where the differences between $E_{0_1}'$ and $E_{0_2}'$ are 0, 5, 10, 20 and 30 mv. respectively. Table II contains the data for calculation purposes and Tables III, IV and V

Table II.

| $\Delta E$ in volts | $e^{-\Delta E\alpha}$ | $e^{\Delta E\alpha}$ | $\Delta E$ in volts | $e^{-\Delta E\alpha}$ | $e^{\Delta E\alpha}$ |
|---|---|---|---|---|---|
| −0·060 | 99·5 | 0·010 | −0·015 | 3·16 | 0·319 |
| −0·055 | 68·1 | 0·012 | −0·013 | 2·71 | 0·369 |
| −0·050 | 46·6 | 0·022 | −0·012 | 2·51 | 0·398 |
| −0·045 | 31·9 | 0·032 | −0·010 | 2·16 | 0·466 |
| −0·040 | 21·6 | 0·047 | −0·008 | 1·85 | 0·541 |
| −0·035 | 14·5 | 0·068 | −0·007 | 1·71 | 0·584 |
| −0·030 | 9·97 | 0·100 | −0·005 | 1·47 | 0·682 |
| −0·025 | 6·82 | 0·146 | −0·004 | 1·36 | 0·736 |
| −0·020 | 4·65 | 0·216 | −0·003 | 1·26 | 0·795 |
| −0·018 | 3·98 | 0·251 | −0·002 | 1·16 | 0·857 |
| −0·017 | 3·69 | 0·271 | −0·001 | 1·08 | 0·926 |

$\alpha$ at 30° = 76·8 using logarithms to the base $e$.

contain the calculated values for the various cases. Calculations were made with an accuracy of one part in the third place. The graphs for Tables III, IV and V are shown in Figs. 1, 2 and 3 respectively. Tables VI and VII offer a convenient summary of all the data.

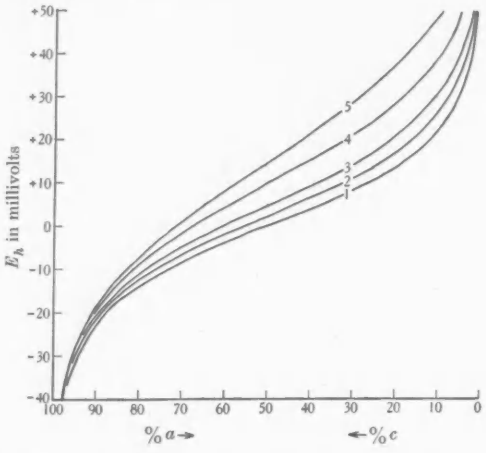



Fig. 1. Variation of $E_h$ with change in the ratio $a/c$, when $b=0$. The numbers refer to the cases worked out in Table III.

Table III. *Variation of* $E_h$ *with change in the ratio* a/c, *when* $b=0$.

| $E_h$ in volts | Case 1 $E_{01}'=0{\cdot}000$ v. $E_{02}'=0{\cdot}000$ v. % a | Case 2 $E_{01}'=0{\cdot}000$ v. $E_{02}'=0{\cdot}005$ v. % a | Case 3 $E_{01}'=0{\cdot}000$ v. $E_{02}'=0{\cdot}010$ v. % a | Case 4 $E_{01}'=0{\cdot}000$ v. $E_{02}'=0{\cdot}020$ v. % a | Case 5 $E_{01}'=0{\cdot}000$ v. $E_{02}'=0{\cdot}030$ v. % a |
|---|---|---|---|---|---|
| −0·030 | 94·6 | 94·6 | 95·0 | 95·0 | 95·0 |
| −0·025 | 91·8 | 92·4 | 92·4 | 93·5 | 93·5 |
| −0·020 | 88·3 | 88·8 | 89·6 | 90·7 | 90·8 |
| −0·015 | 81·8 | 83·7 | 85·0 | 86·5 | 87·4 |
| −0·010 | 73·3 | 76·5 | 78·8 | 81·6 | 82·8 |
| −0·005 | 62·6 | 67·1 | 70·7 | 75·5 | 77·6 |
| 0·000 | 50·0 | 56·0 | 60·9 | 67·7 | 71·4 |
| +0·005 | 37·4 | 44·0 | 50·0 | 59·1 | 64·7 |
| +0·010 | 26·7 | 32·7 | 39·2 | 50·0 | 57·4 |
| +0·015 | 18·2 | 23·5 | 29·3 | 40·8 | 50·0 |
| +0·020 | 11·7 | 16·3 | 21·2 | 32·3 | 42·5 |
| +0·025 | 8·2 | 11·2 | 15·0 | 24·5 | 35·3 |
| +0·030 | 5·4 | 7·6 | 10·4 | 18·4 | 28·6 |
| +0·035 | 3·5 | 5·4 | 7·6 | 13·5 | 22·4 |
| +0·040 | — | 3·5 | 5·0 | 9·3 | 17·2 |
| +0·045 | — | — | — | 6·5 | 12·6 |
| +0·050 | — | — | — | — | 9·1 |

It is clear that the curves for $a/c$ are S-shaped and symmetrical about the mid-point whereas the curves for $b/c$ and $a/b$ are exponential and hence asymmetrical. Inspection of the latter two sets of curves shows that one is the mirror image of the other.

In Tables IV and V negative values for the ratio of $a/b$ or $b/c$ are obtained for certain values of $E_h$. That can only mean that it is impossible to start with a finite ratio and reach that potential level. From Fig. 2 it is apparent that the

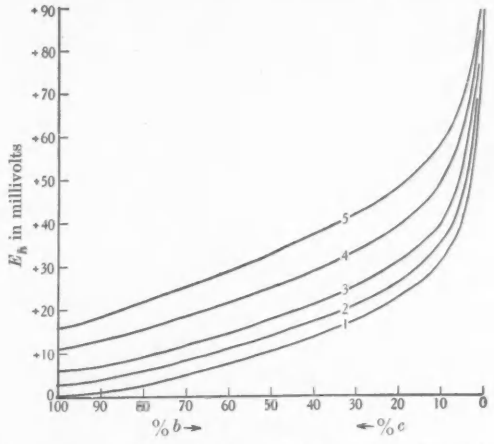



Fig. 2. Variation of $E_h$ with change in the ratio $b/c$, when $a=0$. The numbers refer to the cases worked out in Table IV.

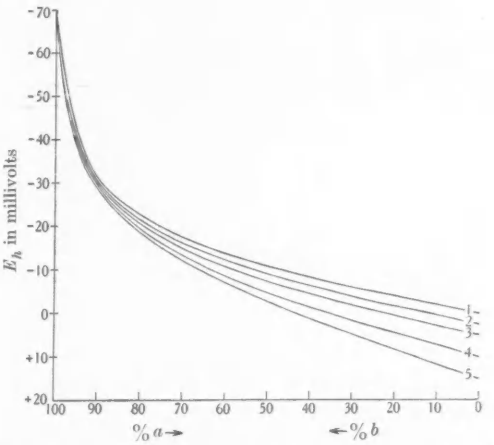



Fig. 3. Variation of $E_h$ with change in the ratio $a/b$, when $c=0$. The numbers refer to the cases worked out in Table V.

curve describing the variation of $E_h$ with $b/c$ may be extended beyond 0 % to the negative side of the abscissa, but the curve then has no physical significance. Similarly the curve for $a/b$ may be extended beyond 100 % to negative percentages but here again the extension of the curve has no physical meaning.

A change of $\Delta(E_{0_2}' - E_{0_1}')$ from 0 to 30 mv. produces a much smaller change in the potential values of $a/b$, $b/c$ and $a/c$ for the two cases. In effect, this means

Table IV. *Variation of* $E_h$ *with change in the ratio* b/c, *when* $a=0$.

| $E_h$ in volts | Case 1 $E_{0_1}'=0{\cdot}000$ v. $E_{0_2}'=0{\cdot}000$ v. % $b$ | Case 2 $E_{0_1}'=0{\cdot}000$ v. $E_{0_2}'=0{\cdot}005$ v. % $b$ | Case 3 $E_{0_1}'=0{\cdot}000$ v. $E_{0_2}'=0{\cdot}010$ v. % $b$ | Case 4 $E_{0_1}'=0{\cdot}000$ v. $E_{0_2}'=0{\cdot}020$ v. % $b$ | Case 5 $E_{0_1}'=0{\cdot}000$ v. $E_{0_2}'=0{\cdot}030$ v. % $b$ |
|---|---|---|---|---|---|
| 0·000 | ∞ | Neg. | Neg. | Neg. | Neg. |
| +0·002 | 90·0 | ,, | ,, | ,, | ,, |
| +0·003 | 84·9 | ,, | ,, | ,, | ,, |
| +0·004 | 79·9 | 92·9 | ,, | ,, | ,, |
| +0·005 | 74·9 | 88·1 | ∞ | ,, | ,, |
| +0·007 | — | — | 91·2 | ,, | ,, |
| +0·008 | — | — | 86·8 | ,, | ,, |
| +0·010 | 53·2 | 65·9 | 78·1 | ∞ | ,, |
| +0·012 | — | — | — | 92·8 | ,, |
| +0·013 | — | — | — | 89·2 | ,, |
| +0·015 | 36·6 | 47·1 | 58·8 | 82·0 | ∞ |
| +0·017 | — | — | — | — | 93·9 |
| +0·018 | — | — | — | — | 90·9 |
| +0·020 | 24·4 | 32·7 | 42·4 | 64·8 | 85·1 |
| +0·025 | 16·2 | 22·3 | 30·0 | 49·5 | 70·5 |
| +0·030 | 10·8 | 15·2 | 20·9 | 36·8 | 57·1 |
| +0·035 | 7·3 | 10·3 | 14·4 | 26·7 | 44·5 |
| +0·040 | 4·8 | 7·0 | 9·9 | 19·2 | 34·6 |
| +0·045 | — | 4·7 | 6·8 | 13·6 | 25·3 |
| +0·050 | — | — | — | 9·5 | 18·4 |
| +0·055 | — | — | — | 6·6 | 13·0 |
| +0·060 | — | — | — | — | 9·3 |
| +0·065 | — | — | — | — | 6·6 |

Table V. *Variation of* $E_h$ *with change in the ratio* a/b, *when* $c=0$.

| $E_h$ in volts | Case 1 $E_{0_1}'=0{\cdot}000$ v. $E_{0_2}'=0{\cdot}000$ v. % $a$ | Case 2 $E_{0_1}'=0{\cdot}000$ v. $E_{0_2}'=0{\cdot}005$ v. % $a$ | Case 3 $E_{0_1}'=0{\cdot}000$ v. $E_{0_2}'=0{\cdot}010$ v. % $a$ | Case 4 $E_{0_1}'=0{\cdot}000$ v. $E_{0_2}'=0{\cdot}020$ v. % $a$ | Case 5 $E_{0_1}'=0{\cdot}000$ v. $E_{0_2}'=0{\cdot}030$ v. % $a$ |
|---|---|---|---|---|---|
| −0·035 | 92·7 | 93·0 | 93·2 | 93·4 | 93·4 |
| −0·030 | 89·2 | 89·7 | 90·1 | 90·5 | 90·7 |
| −0·025 | 83·8 | 84·8 | 85·6 | 86·4 | 87·0 |
| −0·020 | 75·6 | 77·7 | 79·1 | 80·8 | 81·6 |
| −0·015 | 63·4 | 67·3 | 70·0 | 73·3 | 74·7 |
| −0·010 | 46·8 | 52·9 | 57·6 | 63·2 | 65·4 |
| −0·005 | 25·1 | 34·1 | 41·2 | 50·5 | 55·5 |
| −0·004 | 20·1 | — | — | — | — |
| −0·003 | 15·1 | — | — | — | — |
| −0·002 | 10·0 | — | — | — | — |
| 0·000 | 0 | 11·9 | 21·9 | 35·2 | 42·9 |
| +0·001 | Neg. | 7·1 | — | — | — |
| +0·002 | ,, | — | 13·2 | — | — |
| +0·003 | ,, | Neg. | 8·8 | — | — |
| +0·005 | ,, | ,, | 0 | 18·0 | 29·3 |
| +0·007 | ,, | ,, | Neg. | 10·8 | — |
| +0·008 | ,, | ,, | ,, | 7·2 | — |
| +0·010 | ,, | ,, | ,, | 0 | 14·9 |
| +0·012 | ,, | ,, | ,, | Neg. | 9·1 |
| +0·013 | ,, | ,, | ,, | ,, | 6·1 |
| +0·015 | ,, | ,, | ,, | ,, | 0 |

that an experimental error of 1–2 mv. will introduce an inaccuracy of 5–10 mv. in the evaluation of $E_{0_1}'$ and $E_{0_2}'$ respectively.

It is now possible to decide between the alternative of a one-step oxidation of hypoxanthine to uric acid involving four equivalents or a two-step oxidation

involving two equivalents for each step. On the assumption of a one-step oxidation, the variation of the potentials of the hypoxanthine-uric acid system between 90 and 10 % oxidation should be 29 mv., whereas for a two-step oxidation the span of potentials should be between 40 and 60 mv., depending upon the difference in magnitudes of $E_{0_1}'$ and $E_{0_2}'$. Similarly for the xanthine-uric acid system, the span of potentials should be 58 mv. for a one-step oxidation but about 30 mv. for the case of a two-step oxidation.

## Experimental methods.

Xanthine oxidase was prepared from whey according to the directions of Dixon and Kodama [1926]. This preparation retains its activity over a period of months when kept in a vacuum-desiccator. If a more or less clear enzyme solution were required, as in the case of colorimetric studies, the whey preparation was dissolved in water and purified by treatment with charcoal.

Twice recrystallised samples of hypoxanthine, xanthine and uric acid were used in this study. Hypoxanthine and xanthine were dissolved in the minimum quantity of NaOH to yield a 0·03 *M* solution, whereas uric acid was dissolved in a solution containing an equivalent amount of lithium carbonate and a slight excess of alkali. Immediately before each experiment, 5 ml. of the standard solution were brought to the desired $p_H$ and diluted with *M*/5 phosphate buffer to make 10 ml. This manipulation must be very rapid indeed since near neutrality the three purines are rather insoluble and in the strengths used precipitate out within a few minutes unless further diluted with the experimental solutions.

The electrode vessels and procedure have been described in detail in the first paper of this series by Green and Stickland [1934]. There is a point connected with the preparation of agar bridges which may be worth mentioning. Borsook and Schott [1931] have outlined a rather tedious and complicated procedure for filling the capillary tube of the electrode vessel with agar which will withstand a vacuum. The experience of the author has been that by adding 1 g. of finely divided kaolin to 100 ml. of clear filtered agar saturated with KCl, bridges can be made without any undue precautions of slow cooling *etc.* The molten agar is sucked into the capillary tube and cooled immediately. The bridge is very resistant to a vacuum over a period of weeks [Lehmann, 1930].

The electrode vessels were filled with the following solutions: 5 ml. of *M*/5 buffer, 0·5 ml. of 0·001 *M* methyl- or benzyl-viologen, 1 ml. of neutralised purine mixture (0·015 *M*), and 1 ml. of xanthine oxidase solution. Benzylviologen was used between $p_H$ 6 and 7, and methylviologen in the more alkaline range. Both indicators were used at $p_H$ 7·0.

## Experimental results.

Table VIII contains a summary of the observed and theoretical potentials for various $p_H$ levels. Each of the observed values is the mean of from four to six separate determinations.

The following procedure was employed for obtaining the theoretical potentials. First, it was determined by inspection which case best fitted the potentials of HX/U, X/U and HX/X for a given $p_H$. From one observed potential, usually the 5/5 ratio of the HX/U series, all the other eight potentials could easily be evaluated from Table VI. It is noteworthy that given a single potential value for any ratio of two of the purines and knowing the $E_{0_1}'$ and $E_{0_2}'$ values at that

$p_H$, then the potential value for any other ratio, be it HX/X, X/U or HX/U, can easily be determined.

As pointed out previously an experimental accuracy within 1 mv. would be necessary in order to fix the $E_0'$ values with a certainty of 5 mv. But in enzyme systems an accuracy within 2 mv. is difficult to attain. Hence a considerable inaccuracy must be expected in the evaluation of the characteristic constants.

The reasonable agreement between the observed and theoretical values in Table VIII is convincing proof that in the xanthine oxidase system we are dealing

Table VI. *Calculated millivolt changes for various changes in ratio.*

| | HX/U | | | X/U | | | HX/X | | |
|---|---|---|---|---|---|---|---|---|---|
| | 9/1 – 5/5 | 8/2 – 5/5 | 5/5 – 2/8 | 9/1 – 5/5 | 8/2 – 5/5 | 5/5 – 2/8 | 9/1 – 5/5 | 8/2 – 5/5 | 5/5 – 2/8 |
| Case 1 | 23 | 14 | 14 | 9 | 8 | 12 | 21 | 12 | 8 |
| Case 2 | 25 | 15 | 15 | 10 | 8 | 13 | 22 | 13 | 8 |
| Case 3 | 26 | 16 | 16 | 11 | 9 | 13 | 22 | 13 | 9 |
| Case 4 | 29 | 19 | 19 | 12 | 10 | 15 | 24 | 15 | 10 |
| Case 5 | 34 | 23 | 23 | 15 | 11 | 16 | 27 | 16 | 11 |

Table VII. *Calculated* $E_h$ *for 5/5 ratio.*

| | $E_{01}'$ | $E_{02}'$ | HX/U | X/U | HX/X |
|---|---|---|---|---|---|
| Case 1 | 0·000 | 0·000 | 0·000 | +0·011 | −0·012 |
| Case 2 | ,, | +0·005 | +0·0025 | +0·014 | −0·009 |
| Case 3 | ,, | +0·010 | +0·005 | +0·018 | −0·007 |
| Case 4 | ,, | +0·020 | +0·010 | +0·025 | −0·005 |
| Case 5 | ,, | +0·030 | +0·015 | +0·033 | −0·003 |

Table VIII.

| $p_H$ | $E_{01}'$ | $E_{02}'$ | System | | 9/1 | 8/2 | 5/5 | 2/8 |
|---|---|---|---|---|---|---|---|---|
| 6·93 | −0·371 | −0·361 | HX/U | Calc. | — | −0·382 | −0·366 | — |
| | | | | Obs. | — | −0·382 | −0·366 | — |
| | | | X/U | Calc. | — | −0·362 | −0·353 | −0·340 |
| | | | | Obs. | — | −0·362 | −0·349 | −0·335 |
| | | | HX/X | Calc. | — | −0·391 | −0·378 | −0·369 |
| | | | | Obs. | — | −0·393 | −0·381 | −0·372 |
| 7·32 | −0·410 | −0·390 | HX/U | Calc. | — | −0·419 | −0·400 | −0·381 |
| | | | | Obs. | — | −0·422 | −0·400 | −0·380 |
| | | | X/U | Calc. | — | −0·395 | −0·385 | −0·371 |
| | | | | Obs. | — | −0·390 | −0·380 | −0·370 |
| | | | HX/X | Calc. | — | −0·430 | −0·415 | −0·405 |
| | | | | Obs. | — | −0·425 | −0·413 | −0·404 |
| 7·65 | −0·435 | −0·415 | HX/U | Calc. | −0·454 | — | −0·425 | −0·406 |
| | | | | Obs. | −0·454 | — | −0·425 | −0·404 |
| | | | X/U | Calc. | — | — | −0·410 | −0·395 |
| | | | | Obs. | — | — | −0·415 | −0·400 |
| 8·07 | −0·471 | −0·441 | HX/U | Calc. | −0·490 | — | −0·456 | −0·433 |
| | | | | Obs. | −0·484 | — | −0·456 | −0·432 |
| | | | X/U | Calc. | — | −0·455 | −0·444 | — |
| | | | | Obs. | — | −0·452 | −0·440 | — |
| 8·43 | −0·484 | −0·454 | HX/U | Calc. | −0·503 | — | −0·469 | −0·446 |
| | | | | Obs. | −0·497 | — | −0·469 | −0·450 |
| | | | X/U | Calc. | — | −0·462 | −0·451 | −0·435 |
| | | | | Obs. | — | −0·465 | −0·454 | −0·437 |
| 9·10 | −0·512 | −0·482 | HX/U | Calc. | — | — | −0·497 | — |
| | | | | Obs. | — | — | −0·497 | — |
| | | | X/U | Calc. | −0·494 | — | −0·479 | — |
| | | | | Obs. | −0·495 | — | −0·476 | — |

with a two-step oxidation. It is noteworthy that the observed span of potentials for HX/U is approximately twice as much as the ordinary equation predicts, whereas the span of potentials for X/U and HX/X is approximately one-half of the theoretical span for a 2 equivalent system.

In the derivation of the equation for $a/b$, $b/c$ and $a/c$, it was assumed that the following reaction is possible:

$$A + C \rightleftharpoons 2B.$$

If the xanthine oxidase system is to be considered as a case of a two-step oxidation, then the formation of uric acid and hypoxanthine from xanthine and the reverse process must be shown to take place anaerobically in the presence of the enzyme, to an extent determined by the equilibrium constants. The following experiments definitely prove that this dismutation does occur. Xanthine oxidase solution (3 ml.) was incubated with xanthine (3 mg.) anaerobically for 3 hours at 37°. After deproteinisation of the enzyme solution with uranium acetate, the filtrate was tested for uric acid with Benedict's reagent. An intense blue colour developed representing about 1 mg. of uric acid. With boiled enzyme, the test was completely negative. Thus the anaerobic production of uric acid from xanthine is obviously enzymic in nature. When hypoxanthine was incubated with the enzyme under similar conditions, the test was likewise negative. Thunberg tubes were then set up containing different ratios of HX/X and constant amounts of enzyme solution. The results showed that the larger the ratio of HX/X the smaller the amount of uric acid produced and conversely. These observations are in complete accordance with the mathematical theory. Furthermore it was demonstrated that when hypoxanthine and uric acid are incubated anaerobically with the oxidase, a portion of the hypoxanthine and uric acid disappears to form xanthine. The quantitative aspect of this dismutation will be dealt with in a future communication.

Bach and Michlin [1927] claimed that both hypoxanthine and xanthine in presence of the oxidase formed uric acid anaerobically. Wieland [Wieland and Rosenfeld, 1929] was unable to confirm these findings. The fact that hypoxanthine as well as xanthine yielded uric acid indicates that Bach and Michlin were not studying a dismutation but an oxidation—due to leakage of oxygen. The short duration of Wieland's experiments (*ca.* 100 mins.) may explain why the dismutation of xanthine to hypoxanthine and uric acid was not observed. The reaction is rather slow and, unless very active enzyme preparations are employed, at least 6 hours are required for a measurable quantity of uric acid to be formed.


## Summary.

The reversibility of the xanthine oxidase system has been demonstrated by colorimetric and potentiometric methods. The mathematical theory of two-step oxidations involving two equivalents for each step has been developed and evidence presented that the xanthine oxidase system is an example of a two-step oxidation system. The $E_0'$ of the hypoxanthine-xanthine at $p_H$ 7·0 is −0·371 v., and the $E_0'$ of the xanthine-uric acid is −0·361 v. The prediction by the theory of the partial anaerobic formation of uric acid and hypoxanthine from xanthine and the partial anaerobic formation of xanthine from hypoxanthine and uric acid have been confirmed experimentally.


It is a great pleasure to thank Dr Malcolm Dixon for his interest, and Dr F. J. W. Roughton for his assistance with the mathematical portion of the paper.

REFERENCES.

Bach and Michlin (1927). *Ber. deutsch. chem. Ges.* **60**, 82.
Borsook and Schott (1931). *J. Biol. Chem.* **92**, 535.
Cannan (1926). *Biochem. J.* **20**, 827.
Dixon (1926). *Biochem. J.* **20**, 703.
—— and Kodama (1926). *Biochem. J.* **20**, 1104.
Filitti (1933). *Compt. Rend. Acad. Sci.* **197**, 1212.
—— (1934). *Compt. Rend. Acad. Sci.* **198**, 930.
Green and Stickland (1934). *Biochem. J.* **28**, 898.
Lehmann (1930). *Skand. Arch. Physiol.* **58**, 173.
Michaelis (1931). *J. Biol. Chem.* **91**, 369.
—— and Eagle (1930). *J. Biol. Chem.* **87**, 713.
—— and Hill (1933). *J. Gen. Physiol.* **16**, 859.
Wieland and Rosenfeld (1929). *Liebig's Ann.* **477**, 32.

# CCVII. CALOSTEROL, A STEROL PRESENT IN THE MILKY JUICE OF *CALOTROPIS GIGANTEA*.

By KALI PADA BASU and MADHAB CHANDRA NATH.

*From the Biochemical Section, Chemical Laboratory, University of Dacca.*

(*Received June 30th, 1934.*)

In the course of our investigations on the proteolytic enzymes in the milky juice of *Calotropis gigantea* [Basu and Nath, 1933] our attention was drawn to the large amount of ether-soluble substances in the dried juice which gave the Liebermann-Burchard test with acetic anhydride and sulphuric acid. By working up the unsaponifiable matter of the ether-soluble fraction it has been possible to isolate a new sterol which it is proposed to call calosterol. The sterol melts at 202–203° and has therefore a higher melting-point than that of any known sterol [*cf.* Wieland and Gough, 1930] with the exception of cerevisterol [Bills and Honeywell, 1928; Honeywell and Bills, 1932] which melts at 265·3°. Calosterol has the formula $C_{28}H_{44}O$ and thus appears to be isomeric with ergosterol but is dextrorotatory and possesses the high specific rotation in chloroform of $[\alpha]^{24}_{5461} = +100{\cdot}6$. It appears to contain three double bonds. It is not precipitated by digitonin.

### *Preparation and purification.*

85 g. of residue obtained by drying about 425 ml. of the milky juice at the ordinary temperature *in vacuo* were extracted with sodium-dried ether in a Soxhlet apparatus and 33·3 g. of ether-soluble matter obtained. The latter was then saponified with 3 % alcoholic KOH and freed from the insoluble soap by filtration. The alcohol was removed by evaporation on a water-bath and the residue extracted with ether. About 20 g. of unsaponifiable matter were thus obtained. It was crystallised four times from absolute alcohol and from alcohol-benzene mixture (2 : 1). We tried the method of benzoylation and subsequent saponification as adopted by Wieland and Asano [1929] but without success. We therefore repeatedly (six times) crystallised from acetone and each time determined the melting-point and the specific rotation. Ultimately 5·96 g. of seemingly pure crystals of a sterol of M.P. 202–203° and specific rotation $[\alpha]^{24}_{5461} = +100{\cdot}2°$ were obtained. To ensure the greatest possible purity of the sterol it was then benzoylated according to the method recommended by Callow [1931], the sterol being recovered by saponification.

*Calosterol benzoate.* 3·5 g. of the substance were dissolved in 20–25 ml. of redistilled pyridine, cooled in ice and treated with benzoyl chloride drop by drop until a permanent pink colour was obtained. The mixture was allowed to stand for 5 hours when crystals of the benzoate (fraction I) were obtained. These were collected, washed with a little pyridine and the filtrate cooled to −4°, when a second fraction of crystals was obtained. Fractions I and II were separately washed with water and then with alcohol. The aqueous washings from the two

crops were added to the filtrate and the precipitate (fraction III) thus obtained was collected and washed.

| | | | |
|---|---|---|---|
| Fraction I | 2·03 g. | M.P. 237–239° | $[\alpha]^{24}_{5461} = +121{\cdot}0°$ |
| Fraction II | 0·85 g. | M.P. 208–225° | $[\alpha]^{24}_{5461} = +118{\cdot}6°$ |
| Fraction III | 0·61 g. | M.P. 199–206° | $[\alpha]^{24}_{5461} = +114{\cdot}0°$ |

These fractions were then worked up as recommended by Callow until no further alteration in specific rotation took place. The calosterol benzoate thus obtained had M.P. 239–240° and specific rotation $[\alpha]^{24}_{5461} = +121{\cdot}6°$.

(Found: C, 83·74, 83·80; H, 9·67, 9·51 %. $C_{35}H_{48}O_2$ requires C, 84·00; H, 9·60 %.)

*Calosterol.* 1·5 g. of the pure benzoate were saponified with about 70 ml. of 3 % alcoholic KOH for about half an hour. The sterol separated on pouring the mixture into water and was filtered by suction, washed with water and dried in a vacuum. It was then crystallised successively from acetone, methyl alcohol and absolute alcohol. The pure sterol had M.P. 202–203° and a specific rotation of $[\alpha]^{24}_{5461} = +100{\cdot}6°$ in chloroform.

(Found: C, 84·88, 84·74; H, 10·54, 10·64 %. $C_{28}H_{44}O$ requires C, 84·84; H, 11·11 %.)

Calosterol crystallises well from acetone, alcohol and from alcohol-benzene mixture (2 : 1). The sterol is very soluble in ether but only dissolves in about 200 parts of boiling alcohol.

Calosterol is not precipitated by digitonin and thus resembles lanosterol and agnosterol [Lifschütz, 1926] and also cerevisterol [Honeywell and Bills, 1933].

Calosterol is quite stable. Exposed to the air and light of the laboratory for several weeks, it showed no discoloration or change in melting-point.

*Calosterol acetate.* 0·3 g. of the purest calosterol and 5 ml. acetic anhydride were boiled under reflux for 15 mins., cooled, poured into water and filtered. The acetate was washed, dried and crystallised twice from glacial acetic acid; yield 0·2 g. M.P. 211–212° and specific rotation $[\alpha]^{24}_{5461} = +105{\cdot}0°$ in chloroform.

(Found: C, 82·21, 82·12; H, 10·34, 10·15 %. $C_{30}H_{46}O_2$ requires C, 82·19; H, 10·50 %.)

From the analyses of the acetate and the benzoate it is quite clear that calosterol contains one hydroxyl group. It will be seen that the acetate and the benzoate show higher specific rotations than the calosterol itself. While it is unusual for a sterol ester to exhibit greater optical activity than the parent sterol, a similar behaviour has been noted with cerevisterol [Honeywell and Bills, 1932] and *iso*ergosterol [McDonald and Bills, 1930].

*Calosterol hydrate.* The sterol crystallises with one molecule of water of crystallisation from 90 % alcohol and retains this water even *in vacuo*.

(Found: C, 81·03; H, 11·18 %. $C_{28}H_{44}O$, $H_2O$ requires C, 81·16; H, 11·11 %.)

The water could, however, be removed by heating the hydrate to 110° for 2 hours and then drying it *in vacuo*.

With regard to the microanalytical data given above it should be noted that a large number of analyses were made and concordant results obtained. Only a few typical data are given. That the apparatus was functioning correctly was ensured by analysing from time to time a pure sample of cholesterol (Pfanstiehl) when correct values for carbon and hydrogen were obtained.

*Colour reactions of calosterol.*

With the Liebermann-Burchard reagent calosterol gives a reddish violet colour which changes to violet, then quickly to blue and finally to green.

Tortelli-Jaffe's reaction gives a bright yellow colour.

In Salkowski's reaction the chloroform layer is colourless and the sulphuric acid layer is coloured orange-yellow and gives a green fluorescence.

With trichloroacetic acid as well as with chloral hydrate [Rosenheim, 1929] calosterol remained colourless.

With regard to its different colour reactions calosterol thus differs from ergosterol and any other known sterol.

*Unsaturation.*

Using the method of Reindel and Niederlander [1929] experiments were carried out with cholesterol as well as with calosterol. Table I shows the results.

Table I.

| 1 Substance | 2 Wt. of substance g. | 3 $N/10$ Br in $CCl_4$ added ml. | 4 $N/10$ Br in excess ml. | 5 $N/10$ Br transformed to HBr in blank ml. | 6 $N/10$ Br required to saturate double bond ml. | 7 $N/10$ Br required for each double bond ml. | 8 No. of double bonds found |
|---|---|---|---|---|---|---|---|
| Cholesterol | 0·1081 | 16·35 | 7·55 | 1·55 | 7·25 | 5·22 | 1·4 |
| Calosterol | 0·0757 | 34·18 | 17·98 | 3·37 | 12·83 | 3·93 | 3·3 |

The number of double bonds in calosterol thus appears to be three. The slightly higher values obtained might be caused by the bromine solution partially oxidising the OH group and forming hydrobromic acid. Estimation of hydrobromic acid (by adding a solution of potassium iodate and titrating the liberated iodine with $N/10$ sodium thiosulphate) however showed that while the HBr formation in the case of cholesterol was such that by taking that amount into consideration the number of double bonds obtained fell from 1·4 to 1·0, a very large amount of HBr was found in the case of calosterol. This was probably caused by the liberation of HBr by the brominated sterol in presence of water.

*Catalytic hydrogenation.* As catalyst platinum black prepared according to the method of Willstätter and Waldschmidt-Leitz [1921] was employed. The solvent used was glacial acetic acid containing a trace of hydrochloric acid at temperatures of 60–70°. Calosterol was employed in the form of its acetate, parallel experiments being carried out with cholesterol acetate. The amount of catalyst employed was 0·1 g. Table II shows the results.

Table II.

| Substance | Weight in g. | Period of shaking hrs. | Vol. of $H_2$ absorbed at N.T.P. ml. | Vol. of $H_2$ at N.T.P. required to saturate one double bond ml. | No. of double bonds found |
|---|---|---|---|---|---|
| Cholesterol acetate | 0·2012 | 4 | 10·51 | 10·52 | 1·0 |
| Calosterol acetate | 0·0975 | 6·5 | 10·93 | 4·98 | 2·2 |

Thus by catalytic hydrogenation the number of double bonds found is two, which is one less than the number obtained by the method of bromination. Similar behaviour is shown by ergosterol as well as by zymosterol.

Calosterol, $C_{28}H_{44}O$ with three double bonds, thus appears to be isomeric with ergosterol, which has recently been shown by Windaus and Luttringhaus [1932] to have the formula $C_{28}H_{44}O$.

SUMMARY.

Calosterol, a stable sterol isolated from the milky juice of *Calotropis gigantea*, has the formula $C_{28}H_{44}O$. It melts at 202–203° and has a specific rotation in chloroform of $[\alpha]^{24}_{5461} = +100{\cdot}6°$. The sterol is not precipitated by digitonin. The benzoate melts at 239–240° and has $[\alpha]^{24}_{5461} = +121{\cdot}6°$. The acetate melts at 211–212° and has $[\alpha]^{24}_{5461} = +105{\cdot}0°$. The method of bromination gives three double bonds for calosterol while catalytic hydrogenation with platinum black yields only two.

Our best thanks are due to Prof. J. C. Ghosh and Prof. S. N. Bose for their kind interest.

REFERENCES.

Basu and Nath (1933). *J. Ind. Chem. Soc.* (Sir P. C. Ray Jubilee Volume), 107.
Bills and Honeywell (1928). *J. Biol. Chem.* **80**, 15.
Callow (1931). *Biochem. J.* **25**, 79.
Honeywell and Bills (1932). *J. Biol. Chem.* **99**, 71.
—— —— (1933). *J. Biol. Chem.* **103**, 515.
Lifschütz (1926). *Z. physiol. Chem.* **155**, 240.
McDonald and Bills (1930). *J. Biol. Chem.* **88**, 601.
Reindel and Niederlander (1929). *Liebig's Ann.* **475**, 147.
Rosenheim (1929). *Biochem. J.* **23**, 47.
Wieland and Asano (1929). *Liebig's Ann.* **473**, 300.
—— and Gough (1930). *Liebig's Ann.* **482**, 36.
Willstätter and Waldschmidt-Leitz (1921). *Ber. deutsch. chem. Ges.* **54**, 113.
Windaus and Luttringhaus (1932). *Nachr. Ges. Wiss. Göttingen, Math.-Physik. Klasse*, **4**.

# CCVIII. PRUNE AS AN OXIDATION-REDUCTION INDICATOR. ITS SUITABILITY FOR TITRATION OF ASCORBIC ACID.

By JAMES MELVILLE and GEORGE MAXWELL RICHARDSON.

*From the Biochemical Laboratory, Imperial College of Science and Technology, London, S.W. 7.*

(*Received June 28th, 1934.*)

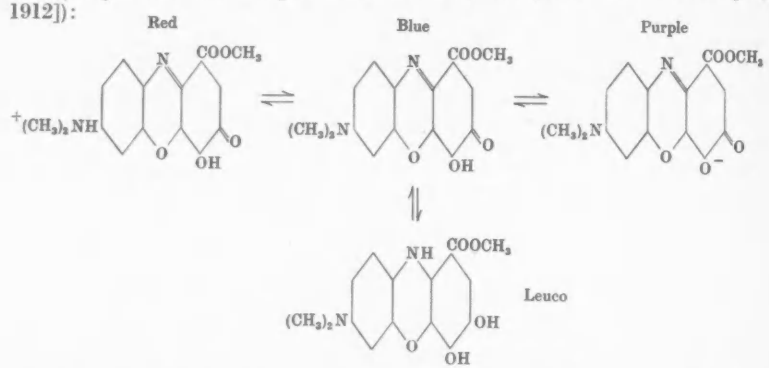
The use of indophenols for oxidative titration of ascorbic acid [Tillmans *et al.*, 1932; Birch *et al.*, 1933; Zilva, 1934] stimulates interest in other positive oxidation-reduction indicators. In particular, the character of the somewhat elusive potentials of ascorbic acid [Green, 1933] suggests that indicators with a range between the indophenols and methylene blue may have practical significance. The oxazine "prune" was found to have such a range and seemed to be a substance of general utility for three reasons: (*a*) prune is readily available as a commercial dyestuff [No. 893, Rowe, 1924], or is easily synthesised from methyl gallate and *p*-nitrosodimethylaniline, (*b*) its potential range is such as to minimise fortuitous oxidation of phenols, *etc.*, by the reagent and to provide the greatest specificity consistent with titration of the somewhat weakly reducing ascorbic acid, and (*c*) its acid-solubility, stability and considerable tinctorial power, as compared with the acid-instability and "rather pale reddish colour" of indophenols [Gibbs *et al.*, 1925] offer considerable advantages where the desired degree of specificity requires the use of acid solutions [Birch *et al.*, 1933]. As other likely indicators (indamines such as toluylene blue [Phillips *et al.*, 1927], aminoindophenols [Cohen and Phillips, 1929], or oxazines such as cresyl blue [Cohen and Preisler, 1931] were chemically unstable in solution, it became desirable to submit the system, prune—reduced prune, to potentiometric investigation.

### *General properties.*

Prune is an intense magenta red in acid solution, a deep blue between $p_H$ 4 and 8 and purple beyond $p_H$ 8. The red material is readily soluble in water, somewhat soluble in aqueous alcohols and insoluble in other solvents. The blue material aggregates slowly in water and precipitates at a rate varying with concentration; it is soluble in chloroform, ethyl acetate, alcohols and benzene, slightly soluble in ether and carbon tetrachloride, insoluble in light petroleum. The purple material is rather unstable but is most soluble in water. From its aqueous solution, prune is readily extractable as the blue material by chloroform or ethyl acetate at any $p_H$, or by benzene at $p_H$ 4–7, yet satisfactory crystallisation from these solvents is hindered by a low temperature coefficient of solubility. The blue material separates from water (after treating the acid solution with sodium acetate) by slow precipitation of an aggregate of clustered needles and amorphous residues, readily soluble in acids. This aggregate is crystallisable from xylene [Kehrmann and Beyer, 1912] as clustered prisms. The red material separates more satisfactorily from 0·1 *M* HCl solution, as prisms of steely lustre, either by salting out or by continuous extraction with butyl alcohol.

It appears that the red material is the cation (hydrochloride), the blue is unionised, and the purple the anion (phenoxide), or in other words that the dissociation constants found at $p_H$ 3·6 and 8·0 represent, respectively, the dissociations of basic and acid groups. This is confirmed by a colour change from red to blue when acetone is added to a solution of prune in phthalate buffer of $p_H$ 3 (evidence of a basic group), and by the reverse change from purple to blue in an ammonia buffer of $p_H$ 9·3 (evidence of an acid group [Richardson, 1934]); in assessing the purple colour, care is needed because of pronounced dichroism in the purple shade.

The magnitudes of these constants are such as are usually associated with anilines ($p_K$ 3 to 5) and phenols ($p_K$ 8 to 10), respectively. Consequently, the following formulae best fit the known properties of prune (including acylation and methylation reactions [Gnehm and Bauer, 1905; Kehrmann and Beyer, 1912]):




It will be observed that the usual oxonium salt formulation is discarded since the basic dissociation common to pyrones is very weak ($p_K$ about 0), and would be responsible only for the third colour change (red-blue) found to occur in prune in approximately 8$M$ acid solutions. Nevertheless, in certain chemical properties the influence of its structural analogy with pyrones is observed. These are the colloidal tendencies of the blue solution, the formation with iodine of a brown sediment decomposable by thiosulphate and the precipitation of acid solutions by ferrocyanides. The influence of its phenolic group is apparent in the formation with ferric chloride in both acid and neutral solution of a blue complex insoluble in butyl alcohol.

Reduced prune is pale yellow in acid or neutral solution, or somewhat brownish alkaline to $p_H$ 9. The acid leuco-compound is most soluble in water and alcohols, the neutral compound in chloroform, alcohols and ether. Aerobic oxidation proceeds slowly in acid solution, much more rapidly in neutral or alkaline solution.

## Experimental.

*Materials.* Prune was prepared as the hydrochloride on account of its ready solubility. Specimens are obtained easily only by salting out, so that purification of commercial materials controlled by analyses was not attempted. Instead, methyl gallate (M.P. 199°) and *p*-nitrosodimethylaniline hydrochloride, both freshly recrystallised, were boiled with methyl alcohol until a spot on filter-paper gave no yellow fringe. The crystalline precipitate of prune hydrochloride was washed with methyl alcohol, dried and thrice salted out of its warm 1 % solution

in 0·05 *M* HCl by bringing the HCl concentration to 0·25 *M* and cooling; it was dried in a vacuum in the presence of NaOH. The resulting prisms gave a positive chloride test after incineration with $HNO_3$. By titration at a quinhydrone electrode, they required one equivalent of NaOH for neutralisation and were thus crystals of a monohydrochloride. A stock solution of 0·004 *M* prune HCl (mol. wt. 350·6) in 0·05 *M* HCl proved admirably stable.

Buffer solutions were of standard type [Clark, 1928; Britton and Robinson, 1931], freshly prepared from recrystallised materials and $CO_2$-free NaOH. As reducing agents, sodium hydrosulphite or titanous salts were unsuitable, and sodium anthrahydroquinone-$\beta$-sulphonate was used. This was prepared by reducing a fresh 0·004 *M* solution of recrystallised sodium anthraquinone-$\beta$-sulphonate with hydrogen and platinised asbestos; the solution was filtered through sintered glass into 4 volumes of deaërated buffer and stored under nitrogen for delivery from a burette. As oxidising agent, recrystallised freshly-dissolved 0·004 *M* benzoquinone, or 0·002 *M* iodine-KI, was employed. Nitrogen and hydrogen were commercial supplies suitably purified by conc. NaOH and hot copper gauze, and conveyed to the titration vessel through copper leads.

*Apparatus.* Titrations were conducted at $30 \pm 0{\cdot}02°$ in an electrically controlled kerosene thermostat. The titration vessel, of standard Clark pattern [1928, p. 301], was in electrical circuit with a saturated calomel half-cell through a saturated KCl bridge of the bulb and goose-neck capillary type. Duplicate gold-plated platinum wire electrodes were used, or, for $p_H$ determination, freshly palladised gold-plated wire electrodes. Calomel electrodes were maintained constantly at 30° and frequently standardised against a hydrogen electrode in 0·05 *M* potassium hydrogen phthalate, of an assumed potential of +0·2386 v. at 30° [Clark, 1928].

*Procedure.* For reduction experiments, the $p_H$ of 25 ml. of the appropriate 0·025 *M* buffer was determined in the titration vessel, and then 1 ml. of 0·004 *M* prune in 0·05 *M* HCl, with sufficient 0·01 *M* NaOH for neutralisation to the buffer $p_H$, was added. Titration from a micro-burette was carried out under nitrogen with approximately 0·0008 *M* anthrahydroquinone-$\beta$-sulphonate in the same buffer.

For oxidation experiments, the stock 0·004 *M* prune was suitably diluted with 0·05 *M* HCl, an aliquot reduced by hydrogen and platinised asbestos and discarded, the main bulk added to the same catalyst, reduced colourless (15 min.), and immediately withdrawn through sintered glass for storage under hydrogen. To 25 ml. of deaërated 0·025 *M* buffer, about 3 ml. of approximately 0·001 *M* reduced prune were usually added, and the $p_H$ was determined. The solution was then titrated under nitrogen either with iodine (avoiding excess), or with a dilution of benzoquinone in the desired buffer, all solutions being deaërated.

In general, oxidising and reducing agents with metallic ions were avoided, because of a marked tendency to co-ordination, exemplified in the behaviour of prune as a mordant dyestuff. Since this has necessitated the use of catalytically reduced reagents, their concentration has been known only approximately. Consequently, $E_0'$ values have been separately determined at each $p_H$ by a complete titration, and each value represents the mean constant for the curve. During the titration, duplicate electrodes rarely disagreed by more than 0·0001 v.

*Potentiometric results.*

Admirable potentiometric data were obtained on the acid side of $p_H$ 4, and indeed were satisfactory as far as $p_H$ 10, since the prune solutions employed (0·00015 *M*), though richly coloured, were sufficiently dilute to avoid any

considerable aggregation during titration. In presence of borates and glycine, however, reproducibility of $E_0'$ values was hindered by co-ordination and aggregation effects. Decomposition of prune to dimethylaniline [Kehrmann and Beyer, 1912] begins above $p_H$ 10.

In Table I data for two oxidations of reduced prune by benzoquinone are given, while Table II presents two reductions of prune by anthrahydroquinone-$\beta$-sulphonate. It seems likely from Table II that our thrice crystallised material still contained some 5 % of an impurity, 0·08 v. more negative than prune at $p_H$ 2·4. Possibly this was the demethylated dyestuff, gallocyanine [Michaelis and Eagle, 1930], or an oxazone [*cf.* cresyl blue, Cohen and Preisler, 1931]. In any event its amount was not increased above that for a fresh (2 hours) solution by keeping for 10 days in 0·05 *M* HCl, and its influence on Tables I and II is negligible except at the negative extremes.

The relation of $E_0'$ to $p_H$ is given in Table III, adopted values of the dissociation constants being confirmed by hydrogen electrode titration of reduced prune, and by colorimetric examination of prune. The electrode equation from which calculation is made [*cf.* Cohen and Preisler, 1931] is as follows:

$$E_h = E_0 - 0{\cdot}03006 \log \frac{S_r}{S_o} - 0{\cdot}03006 \log \frac{K_{ob}K_{oa} + K_{ob}[H^+] + [H^+]^2}{K_{rb}K_{ra}[H^+]^2 + K_{rb}[H^+]^3 + [H^+]^4}$$

where $E_h + 0{\cdot}03006 \log \frac{S_r}{S_o} = E_0'$. It is based on the assumption of the following hydrion dissociations:

$$Ox^+ \xrightarrow{K_{ob}} Ox \xrightarrow{K_{oa}} Ox^-, \text{ and } Red^+ \xrightarrow{K_{rb}} Red \xrightarrow{K_{ra}} Red^- \xrightarrow{K_{ra_2}} Red^{--} \xrightarrow{K_{ra_3}} Red^{---}$$

of which $K_{ra_3}$ is negligible, and $K_{ra_2}$ is also inoperative in the range described

Table I. *Titration of reduced prune with benzoquinone.*

(*a*) $p_H$ 2·886 — 35 ml. 0·025 *M* phthalate, 0·0008 *M* benzoquinone — 7 ml. 0·001 *M* reduced prune

(*b*) $p_H$ 6·938 — 25 ml. 0·025 *M* phosphate, 0·0008 *M* benzoquinone — 3 ml. 0·001 *M* reduced prune

| | Titre (ml.) | $0{\cdot}03006 \log \frac{S_r}{S_o}$ | $E_h$ | $E_0'$ |
|---|---|---|---|---|
| (*a*) | 0·47 | +0·038 | +0·3172 | +0·355 |
| | 0·71 | 0·0318 | 0·3220 | 0·3538 |
| | 1·03 | 0·0264 | 0·3272 | 0·3536 |
| | 1·31 | 0·0228 | 0·3310 | 0·3538 |
| | 1·85 | 0·0173 | 0·3360 | 0·3533 |
| | 2·47 | 0·0123 | 0·3410 | 0·3533 |
| | 3·08 | 0·0080 | 0·3454 | 0·3534 |
| | 3·86 | +0·0032 | 0·3503 | 0·3535 |
| | 4·58 | −0·0011 | 0·3546 | 0·3534 |
| | 5·37 | 0·0059 | 0·3593 | 0·3534 |
| | 6·17 | 0·0112 | 0·3649 | 0·3537 |
| | 6·90 | 0·0169 | 0·3706 | 0·3537 |
| | 7·57 | 0·0238 | 0·3771 | 0·3533 |
| | 7·91 | 0·0286 | 0·3813 | 0·3527 |
| | 8·24 | 0·035 | 0·3864 | 0·351 |
| | 8·79 (end-point) | | | |
| (*b*) | 0·50 | +0·028 | +0·0283 | +0·056 |
| | 1·20 | 0·0140 | 0·0462 | 0·0602 |
| | 2·00 | +0·0039 | 0·0560 | 0·0599 |
| | 2·70 | −0·0039 | 0·0637 | 0·0598 |
| | 3·20 | 0·0099 | 0·0695 | 0·0596 |
| | 3·70 | 0·0171 | 0·0770 | 0·0599 |
| | 4·30 | 0·031 | 0·0870 | 0·056 |
| | 4·70 (end-point) | | | |

Table II. *Titration of prune with anthrahydroquinone-β-sulphonate.*

(*a*) $p_H$ 2·402. 25 ml. 0·025 *M* phthalate. 1 ml. 0·004 *M* prune in 0·05 *M* HCl. 2·55 ml. 0·014 *M* NaOH. 0·0008 *M* (approx.) anthrahydroquinone-β-sulphonate buffered at $p_H$ 2·4.

(*b*) $p_H$ 7·698. 25 ml. 0·025 *M* barbitone. 1 ml. 0·004 *M* prune in 0·05 *M* HCl. 3·9 ml. 0·014 *M* NaOH. 0·0008 *M* (approx.) anthrahydroquinone-β-sulphonate buffered at $p_H$ 7·7. Correction, 0·08 ml.*

| | Titre ml. | Titre (corr.) ml. | $0{\cdot}03006 \log \frac{S_r}{S_o}$ | $E_h$ | $E_0'$ |
|---|---|---|---|---|---|
| (*a*) | 0·25 | — | −0·0408 | +0·4253 | +0·3845 |
| | 0·50 | — | 0·0312 | 0·4162 | 0·3850 |
| | 1·00 | — | 0·0208 | 0·4062 | 0·3854 |
| | 1·70 | — | 0·0119 | 0·3973 | 0·3854 |
| | 2·52 | — | −0·0040 | 0·3891 | 0·3851 |
| | 3·31 | — | +0·0030 | 0·3820 | 0·3850 |
| | 4·30 | — | 0·0127 | 0·3723 | 0·3850 |
| | 5·01 | — | 0·0222 | 0·3629 | 0·3851 |
| | 5·50 | — | 0·033 | 0·3532 | 0·386 |
| | 5·93 (end-point) | — | — | — | — |
| | 5·96 | — | — | 0·331 | — |
| | 6·06 (colourless) | — | — | 0·318 | — |
| | 6·15 | — | — | 0·302 | — |
| | 6·27 | — | — | 0·234 | — |
| (*b*) | 0·25 | 0·17 | −0·0448 | +0·0669 | +0·0221 |
| | 0·50 | 0·42 | 0·0324 | 0·0543 | 0·0219 |
| | 1·00 | 0·92 | 0·0200 | 0·0427 | 0·0219 |
| | 1·72 | 1·64 | 0·0109 | 0·0330 | 0·0221 |
| | 2·50 | 2·42 | −0·0028 | 0·0249 | 0·0221 |
| | 3·50 | 3·42 | +0·0070 | 0·0153 | 0·0223 |
| | 4·30 | 4·22 | 0·0164 | +0·0057 | 0·0221 |
| | 4·80 | 4·72 | 0·0249 | −0·0025 | 0·0224 |
| | 5·19 | 5·11 | 0·0366 | 0·0116 | 0·025 |
| | — | 5·42 (end-point) | — | — | — |
| | 5·60 | — | — | 0·024 | — |
| | 5·91 | — | — | 0·033 | — |

* An artefact due to aggregation of oxidant in the early stage of titration in neutral solution.

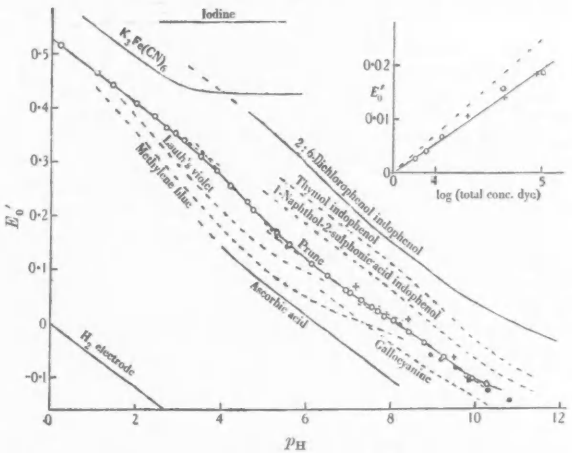



Fig. 1. $E_0'$-$p_H$ curves for prune and adjacent dyes. Data of Table III ○; with borate ×; with glycine •.

*Inset.* $E_0'$-log (concentration) curves for prune.
($E_0'$ in 0·00025 *M* solution adopted as zero potential.)
$p_H$ 4·43 ○; $p_H$ 1·76 ×; Nile blue at $p_H$ 4·88 and 1·44 - - - -.

($p_{K_{ra_2}}=10$). The oxidation-reduction is the ordinary two-electron transfer, $Ox^{+} \rightleftharpoons Red^{-}$. In Fig. 1, the $E_0'$-$p_H$ curve for prune is compared with adjacent indicator systems.

Obviously, prune provides a valuable indicator intermediate between Lauth's violet and the indophenols over the $p_H$ range 2–7, for which region there has hitherto been no indicator chemically stable in solution. At ordinary biological

Table III. *Relation of* $E_0'$ *to* $p_H$.

Approx. 0·00015 *M* prune. $E_0=+0{\cdot}528$ v. $p_{K_{ob}}=3{\cdot}60$, $p_{K_{rb}}=5{\cdot}35$, $p_{K_{ra}}=7{\cdot}45$, $p_{K_{oa}}=8{\cdot}05$.

| $p_H$ | Reagent | Buffer | $E_0'$ (observed) | $E_0'$ (calculated) | Difference |
|---|---|---|---|---|---|
| 0·200 | Anthr.-β-sulph. | HCl | +0·515 | +0·516 | −0·001 |
| 1·046 | ,, | ,, | 0·465 | 0·465 | 0·000 |
| 1·417 | Iodine | HCl-phosphate | 0·442 | 0·443 | −0·001 |
| 1·980 | ,, | ,, | 0·408 | 0·409 | −0·001 |
| 2·402 | Anthr.-β-sulph. | 0·025 *M* phthalate | 0·385 | 0·383 | +0·002 |
| 2·683 | ,, | ,, | 0·363 | 0·365 | −0·002 |
| 2·886 | Quinone | ,, | 0·353 | 0·352 | +0·001 |
| 3·097 | Anthr.-β-sulph. | ,, | 0·340 | 0·338 | +0·002 |
| 3·488 | Iodine | ,, | 0·310 | 0·311 | −0·001 |
| 3·870 | Anthr.-β-sulph. | ,, | 0·283 | 0·282 | +0·001 |
| 4·195 | ,, | ,, | 0·255 | 0·256 | −0·001 |
| 4·590 | ,, | ,, | 0·225 | 0·223 | +0·002 |
| 4·727 | Iodine | 0·036 *M* phthalate | 0·210 | 0·212 | −0·002 |
| 5·290 | Anthr.-β-sulph. | 0·025 *M* phthalate | 0·168 | 0·167 | +0·001 |
| 5·595 | ,, | ,, | 0·146 | 0·145 | +0·001 |
| 6·108 | ,, | 0·025 *M* phosphate | 0·110 | 0·111 | −0·001 |
| 6·492 | ,, | ,, | 0·088 | 0·087 | +0·001 |
| 6·938 | Quinone | ,, | 0·060 | 0·061 | −0·001 |
| 7·032 | Anthr.-β-sulph. | ,, | 0·056 | 0·056 | 0·000 |
| 7·298 | ,, | 0·025 *M* barbitone | 0·042 | 0·042 | 0·000 |
| 7·550 | ,, | 0·025 *M* phosphate | 0·029 | 0·029 | 0·000 |
| 7·698 | ,, | 0·025 *M* barbitone | 0·022 | 0·021 | +0·001 |
| 7·852 | ,, | 0·025 *M* phosphate | 0·012 | 0·013 | −0·001 |
| 8·058 | ,, | 0·025 *M* barbitone | +0·004 | +0·003 | +0·001 |
| 8·418 | ,, | ,, | −0·017 | −0·016 | −0·001 |
| 8·795 | ,, | ,, | 0·037 | 0·037 | 0·000 |
| 9·261 | ,, | 0·025 *M* carbonate | 0·064 | 0·064 | 0·000 |
| 9·792 | ,, | ,, | 0·099 | — | — |
| 9·931 | ,, | ,, | 0·104 | — | — |
| 10·265 | ,, | ,, | 0·115 | — | — |

values of $p_H$ it exists as a neutral molecule as distinct from Lauth's violet, the salt of a base. It has therefore considerable value, at least until replaced by a more soluble indicator of similar range. Its disadvantages arise from its colloidal tendencies and are apparent (*a*) in the dependence of $E_0'$ on the concentration of dyestuff (inset in Fig. 1) which is of the same magnitude as for the oxazine, Nile blue [Cohen and Preisler, 1931], and (*b*) in the eventual aggregation of the neutral solution to a precipitate, which is not serious as it is delayed 12–24 hours in concentrations less than 0·0001 *M*. Prune (blue) has a slightly greater tinctorial power than either methylene blue or 2:6-dichlorophenolindophenol (blue).

It is interesting to note that methylation of the free carboxyl group of gallocyanine [Michaelis and Eagle, 1930] to produce prune, gives a more positive indicator, in which the dissociation of phenolic groups is, as expected, considerably enhanced.

*Titrimetric estimation of ascorbic acid.*

Oxidative titration as an approximate measure of ascorbic acid assumes that only ascorbic acid in its reduced condition (or capable of reduction [Tillmans *et al.*, 1932; Zilva, 1934]) is possessed of antiscorbutic activity. Likewise, it is also

necessary in biological fluids that only ascorbic acid, among reducing substances present, shall be titrated; examples of this difficulty are multiplying. Now, should substances more strongly reducing than ascorbic acid be present, it is clearly impossible for direct oxidative titration to give reliable results. On the other hand, if these substances are weakly reducing, then increase of specificity will follow the use of as negative an oxidising agent as possible, a development hitherto hindered by lack of any suitable reagent [Birch *et al.*, 1933].

It was therefore desirable to discover how far the use of 2:6-dichlorophenol-indophenol could now be bettered, or how far oxidative methods were incapable of direct improvement. A feature of the oxidation of ascorbic acid is its lack of thermodynamic reversibility [Green, 1933], whence there may be expected a somewhat empirical behaviour with oxidising agents or a considerable time lag in the occurrence of possible reactions (see note, p. 1574). For example, the reduction of methylene blue by ascorbic acid is possible anaerobically at $p_H$ 7 [Green, 1933], but is insufficiently facile for adaptation to titrimetric ends.

In a study therefore of the reaction of lemon juice with selected dyes, results were obtained as follows:

(*a*) The degree of reduction at given $p_H$ in a test-tube at room temperature was estimated by adding 1 ml. 0·001 *M* dye (0·4 equiv.) to a mixture of 3 ml. 0·05 *M* buffer + 1 ml. lemon juice + requisite NaOH + water to 9 ml., and comparing with control colour dilutions. *Methylene blue* under these conditions became slowly reduced to about 50 % at $p_H$ 1, but only slightly reduced at $p_H$ 7. *Lauth's violet*, acid to $p_H$ 4, reached a steady state in 20 mins. at about 70 to 80 % reduction, but alkaline to $p_H$ 5 showed only slight visible reduction. *Gallocyanine* showed optimum reduction (70 to 80 %) at $p_H$ 3 and 4 in 15 mins.; at $p_H$ 2 reduction appeared steady at 50 % in 30 mins., but alkaline to $p_H$ 5 reduction was only slight. *Prune* was fully reduced between $p_H$ 2 and 6, the maximum rate (10 mins.) being at $p_H$ 3 and 4; at $p_H$ 1 and $p_H$ 9 reduction was incomplete or else very slow. *1-Naphthol-2-sulphonic acid indophenol*, like prune, was reduced only slowly. *Thymolindophenol* and *2:6-dichlorophenolindophenol* were reduced almost immediately.

(*b*) In developing these findings for titrimetric adaptation, they were repeated in hot solution under $CO_2$, using 1 ml. 0·001 *M* dye added to 1 ml. lemon juice suitably neutralised. *Lauth's violet* now reacted in 2 mins., reaching (in absence of oxygen) 90 % reduction at $p_H$ 1, and 95 to 98 % at $p_H$ 3 and 4. *Gallocyanine* at $p_H$ 4 became rapidly 80 to 90 % reduced. *Prune* was almost immediately reduced at $p_H$ 3, and reduced in 5 mins. at $p_H$ 1. At $p_H$ 4, reduction was immediate but not quite complete. In general, the dyes appeared to reach an equilibrium with the lemon juice, the equilibrium being more slowly attained but favouring greater reduction the cooler the solution. Using ascorbic acid itself in various instances, no essential difference was found. It seems therefore that the behaviour of the dyes examined is mutually quite consistent with their positions in Fig. 1, but that the reactivity of ascorbic acid is such that prune is as weak an oxidising agent as can suitably be used (see note, p. 1574).

*Procedure.* The following procedure should therefore provide optimum specificity in titration.

Place an aliquot of the extract for examination in a short test-tube under an atmosphere of $CO_2$ for titration by 0·0004 *M* prune—from the stock 0·004 *M* solution in 0·05 *M* HCl. After a small addition of prune, adjust the $p_H$ to about 3 (a bluish red) with acid or alkali, heat almost to boiling, and continue the titration under a stream of $CO_2$. The prune solution of course must be substantially free of all more negative coloured impurities if sharp titration is to be attained.

Under these conditions, prune was not reduced by quinol or pyrogallol, and only slowly by cysteine (especially in the presence of cyanide—cation). The reaction of lemon juice with positive dyes was not significantly catalysed by iron or inhibited by cyanide. Yet it was interesting that well-boiled lemon juice (which did not reduce prune) was found to give immediate reduction on addition of ferrous iron; this however was due to enhancement of the reducing intensity of the iron consequent upon co-ordination. Potassium ferricyanide may be used to titrate lemon juice at $p_H$ 4 with prune as indicator, though titration is tedious since the solution may not be warmed.

*Comparison of titration methods.* Although the above technique is thus less straightforward than for 2:6-dichlorophenolindophenol, the use of prune has the practical advantages (*a*) that its acid tinctorial power is roughly three times that of acid indophenol, and (*b*) that it is satisfactorily stable under the conditions of test. Now, even though Birch *et al.* [1933] note that their titration in 5 % tricholoroacetic acid must be accomplished in 2 mins., the point still needs strong emphasis. It was found, using dilutions of 2:6-dichlorophenolindophenol at $p_H$ 4 as colour controls, that 0·0002 *M* indophenol in 4 % trichloroacetic acid was half decolorised in 50 seconds, 75 % in 100 seconds and fully in 5 mins., simply as a result of acid decomposition of the dye. Similar results were obtained for other strong acids and other indophenols. There was however considerable improvement with more dilute or weaker acids. The rate of fading grew insignificant only when acidity was less than $p_H$ 2·5 (*e.g.* using acetic acid). In contrast, dilute solutions of prune are stable for many hours under the experimental conditions at $p_H$ 3 and $p_H$ adjustment is easy because prune is its own indicator.

In Table IV, comparison of various titration methods is made on fresh, centrifuged lemon juice (kept in $CO_2$), using titrants standardised against each other and ferric iron *via* a solution of anthrahydroquinone-$\beta$-sulphonate. Prune gives titres which are consistently smaller than those by other reagents. The disparity is not due to the presence of citrates, nor is it the result of hot titration

Table IV. *Comparison of titration methods on lemon juice.*

(*a*) Titres as ml./ml. lemon juice.

| | 0·0040 *M* $K_3Fe(CN)_6$ | 0·0020 *M* 2:6-Indo.* (in air) | 0·0020 *M* 2:6-Indo.* (in $CO_2$) | 0·0018 *M* prune (hot, in $CO_2$) |
|---|---|---|---|---|
| Sample A, $p_H$ 4 | 1·38–1·41 | 1·37 | 1·43 | 1·31 |
| | 1·38–1·40 | 1·39 | 1·43 | 1·33 |
| | — | 1·38 | — | — |
| | 0·001 *M* iodine | 0·00043 *M* 2:6-Indo. | | 0·00041 *M* prune |
| Sample B, $p_H$ 4 | — | 5·60 | 5·76 | 4·8–5·0 |
| | | | 5·74 | |
| | | | 5·82 (hot) | |
| $p_H$ 3 | 6·60 | — | 5·76 (hot) | 5·00 |
| | | | | 4·96 |
| 0·005 *M* cysteine in sample B | — | 5·84 | — | 5·6 (fades) |
| | | | | 5·5 (fades) |

(*b*) Titratable "ascorbic acid" in lemon juice.

| | Iodine | $K_3Fe(CN)_6$ | 2:6-Indo. | Prune |
|---|---|---|---|---|
| Sample A | — | 0·0028 *M* | 0·0028 *M* | 0·0024 *M* |
| Sample B | 0·0033 *M* | — | 0·0025 | 0·0020 |
| Data of Johnson [1933] | 0·0034 | — | 0·0024 | — |
| ,, | 0·0051 | — | 0·0041 | — |

* 2:6-Dichlorophenolindophenol.

(*cf.* indophenol titres). Consequently it seems certain that prune, by reason of its more negative potentials, is able to eliminate from titration certain substances to which the other reagents are sensitive.

On the other hand, a disadvantage of prune titration is that its longer duration makes it more susceptible to interference by cysteine in bulk. Moreover, prune, even though less easily reduced than indophenols, will still be reducible by dialuric acid, lactoflavine, various natural pigments *etc.* [Bierich *et al.*, 1934; Barron and Hastings, 1934; Friedheim, 1934, Table VI]. Likewise, it is reducible by the brain extract of Young and Mitolo [1934] (to whom we are indebted for a sample). Unfortunately, then even though prune is as negative a titrant as it is convenient to use, it cannot offer really emphatic advantages in specificity.

In conclusion therefore we find in prune a deeply coloured, comparatively stable, readily procured titrating agent, which allows the maximum possible specificity in the direct oxidative titration of ascorbic acid. Its rather negative potential range necessitates its use in an inert atmosphere and in hot solution to accelerate the reaction. Although it thus represents the limit to which oxidising agents may be weakened in titrating ascorbic acid, it is not weak enough to eliminate various other biological reducing agents from the titration. In consequence, it offers a slight though not emphatic advantage over 2:6-dichlorophenolindophenol, but further emphasises that direct oxidative titration of "ascorbic acid" in biological fluids must be regarded with suspicion.

## Summary.

The electrode equation for the oxazine dye, prune [Rowe, 1924, No. 893], has been determined for the $p_H$ range 0 to 10. As an oxidation-reduction indicator, it usefully occupies the region intermediate between Lauth's violet and the indophenols ($E_0 = +0{\cdot}528$ v.). It is most useful in acid ranges, since neutral solutions show colloidal tendencies and become aggregated on keeping.

As a reagent for the titration of ascorbic acid (warmed at $p_H$ 3 under $CO_2$), it represents the limit to which oxidising agents may be weakened without hindering successful titration. It is however not weak enough to eliminate various other biological reducing agents from the titration. Oxidative titration methods must continue to be regarded as having only tentative significance.

The authors wish to thank Prof. A. C. Chibnall for providing every facility for their work. One of us (J. M.) is indebted to the University of New Zealand for a scholarship, the other (G. M. R.) to the Department of Scientific and Industrial Research for a research grant.

## REFERENCES.

Barron and Hastings (1934). *J. Biol. Chem.* **105** (Proc.), vii.
Bierich and Rosenbohm (1934). *Z. physiol. Chem.* **223**, 136.
—— and Lang (1934). *Z. physiol. Chem.* **223**, 180.
Birch, Harris and Ray (1933). *Biochem. J.* **27**, 590.
Borsook and Keighley (1933). *Proc. Nat. Acad. Sci.* **19**, 875.
Britton and Robinson (1931). *J. Chem. Soc.* 1459.
Clark (1928). The determination of hydrogen ions. (London.)
Cohen and Phillips (1929). *U.S. Public Health Reports*, Supplement 74.
—— and Preisler (1931). *U.S. Public Health Reports*, Supplement 92.
Emmerie (1934). *Biochem. J.* **28**, 268.

Friedheim (1934). *Biochem. J.* **28**, 184.
Fruton (1934). *J. Biol. Chem.* **105**, 79.
Gibbs, Cohen and Cannan (1925). *U.S. Public Health Reports*, **40**, 649.
Gnehm and Bauer (1905). *J. prakt. Chem.* **72**, 249.
Green (1933). *Biochem. J.* **27**, 1044.
Johnson (1933). *Biochem. J.* **27**, 1287.
Kehrmann and Beyer (1912). *Ber. deutsch. chem. Ges.* **45**, 3338.
Martius and Euler (1934). *Biochem. Z.* **271**, 9.
Michaelis and Eagle (1930). *J. Biol. Chem.* **87**, 713.
Phillips, Clark and Cohen (1927). *U.S. Public Health Reports*, Supplement 61.
Richardson (1934). *Proc. Roy. Soc. Lond.* B **115**, 133.
Rowe (1924). Colour index. (Bradford.)
Tillmans, Hirsch and Dick (1932). *Z. Untersuch. Lebensmitt.* **63**, 267.
—— —— and Jackisch (1932). *Z. Untersuch. Lebensmitt.* **63**, 276.
Young and Mitolo (1934). *Nature*, **133**, 572.
Zilva (1934). *Biochem. J.* **28**, 663.

*Note added July 26th, 1934.* Borsook and Keighley [1933] hold that the oxidation of ascorbic acid is thermodynamically reversible, measurements being confused by negative drifts due to instability of dehydroascorbic acid. Their potential values (positive of course to those of Fig. 1) overlie, alkaline to $p_H$ 5, those for Lauth's violet. This view accords well with our indicator studies and still accounts for the delayed reduction of more negative indicators (2 to 18 days [Fruton, 1934]). Interference with the indophenol titration by cysteine has been considered by Emmerie [1934], and with special reference to $p_H$ control, by Martius and Euler [1934].

# CCIX. THE FORMATION OF HYPONITROUS ACID AS AN INTERMEDIATE COMPOUND IN THE BIOLOGICAL OR PHOTOCHEMICAL OXIDATION OF AMMONIA TO NITROUS ACID.

## I. CHEMICAL REACTIONS.

By ALEXANDER STEVEN CORBET.

*From the I.C.I. Agricultural Research Station, Jealotts Hill, Bracknell, Berks.*

(*Received July 20th, 1934.*)

That the oxidation of ammonia to nitric acid is a transformation essential to the maintenance of life has long been realised, but our knowledge of the mechanism of the process is still far from complete. The change is of vital importance in the soil and it is from the viewpoint of the soil chemist that the subject is considered in the present paper. Although ammonium sulphate has for long ranked as a very popular fertiliser, the changes which it undergoes in the soil are still but imperfectly understood.

Since the discovery that nitrification was effected by bacteria [Schloesing and Müntz, 1877; 1878; 1879], it has been tacitly assumed that nitrification in the soil is entirely a biological process: it is true that nitrite formation from ammonium salts by ultra-violet radiation was reported over 20 years ago [Berthelot and Gaudechon, 1911] but it is only within the last year or two that evidence has been adduced to show that chemical forces may play an important rôle in nitrification.

The literature relevant to the biological aspects of the subject has been recently reviewed by Barritt [1933] and to this paper reference should be made.

Nitrite-forming organisms, other than the well-known autotrophic *Nitrosomonas*, have been described on several occasions, but their activities appear to be very limited compared with those of *Nitrosomonas*, and there seems every reason to believe that this organism is the most important factor in the biological production of nitrites (and hence of nitrates) from ammonia.

No compounds of composition intermediate between ammonia and nitrous acid have been detected hitherto during the oxidation processes, but it is clear that such compounds must have an existence, even if only a transient one, and there is evidence to show that their existence cannot be as evanescent as is usually assumed. Some work carried out by Beesley [1914] on the biological oxidation of ammonium salts showed that a considerable disappearance of nitrogen, amounting to nearly half the total nitrogen present, occurred in the oxidation of ammonia to nitrous acid; this disappearance was not attributable to the escape of gaseous nitrogen compounds, for most of the nitrogen was finally accounted for in the form of nitrate. That the missing nitrogen in Beesley's experiments was present in the form of some intermediate compound there can be little doubt.

The considerations mentioned above suggested that the whole subject of nitrification merited detailed investigation.

## Scheme of work.

The present investigation is concerned with the oxidation of ammonia to nitric acid by such processes as can take place in the soil. It soon became evident that the transformation of ammonia to nitric acid *in natura* is not solely a microbiological process, it being possible for the complete change to be effected by a chemical mechanism.

The nitrification of ammonia is most conveniently considered as comprising the two stages:

$$\text{(A)} \quad NH_3 \rightarrow HNO_2.$$

$$\text{(B)} \quad HNO_2 \rightarrow HNO_3.$$

Both transformations can be effected by bacteria but both can take place in the absence of living organisms.

In temperate regions the change (A) is carried out in the soil primarily by the agency of *Nitrosomonas*, for the other nitrite-forming organisms appear to have little practical significance. This first stage can be effected also by the action of light and it is now evident that this photochemical change has more than a theoretical significance.

The second stage (B) may be carried out as the result of the activities of the nitrate-forming *Nitrobacter* but it is now clear that, in acid solution, the transformation is catalysed by the hydrogen ion. Evidently, therefore, in acid soils, the oxidation of nitrous acid to nitric acid must take place chemically so that no accumulation of nitrite is possible, and it is significant that many writers have reported the scarcity of *Nitrobacter* in soil compared with *Nitrosomonas*. In many of the biological oxidations carried out in this research, in which soil solution was used as the inoculum and the media were neutral or slightly alkaline, the oxidation did not proceed beyond the formation of nitrite.

It is interesting to note that none of the changes described here entails any loss of nitrogen in a gaseous form: this, however, does not preclude the possibility that the course of denitrification may be accompanied by losses of nitrogen as nitrous oxide or elementary nitrogen.

The present paper is concerned with the chemical changes occurring during nitrification, whilst the transformations effected by microbiological agency will be dealt with subsequently.

Special attention has been paid to the question of intermediate compounds, for the results obtained by Beesley show very clearly that at least one intermediate compound must be capable of a stable existence. It has been tentatively suggested by different writers that hydroxylamine and hyponitrous acid may occur as intermediate compounds during the process of nitrification but no evidence has been adduced in support of these views. It is true that these compounds are the most stable known of the possible intermediates, but it seems improbable that hydroxylamine plays a significant part, for its presence in solution with nitrite entails a loss of nitrogen in some gaseous form.

Recently hydroxylamine has been reported as an intermediate compound during the process of denitrification [Blom, 1928; Lindsay and Rhines, 1932] but this does not mean that it is probably present during the reverse change.

## Discussion.

### *Hyponitrous acid.*

One of the most interesting facts emerging from the present investigation is the identification of hyponitrous acid as an intermediate compound in the course of the oxidation of ammonia to nitrous acid, both by microbiological

agency and by the action of light. Little is known concerning the chemical nature of this compound but there appears to be no doubt that the calcium salt has a stable existence under the experimental conditions which obtained. The detection and estimation of hyponitrites is not easy: on standing, an aqueous solution of the calcium salt is decomposed in part, if not wholly, to nitrous oxide. This being so, any hyponitrite-nitrogen escapes detection and estimation during routine determinations of ammonia, nitrite and nitrate. Nevertheless, the stable existence of calcium hyponitrite in presence of certain concentrations of other salts is by no means precluded.

The formation of the greenish yellow, insoluble silver salt was the only qualitative test for hyponitrites found in the literature but this test is of little value in presence of large amounts of nitrites and other salts. An attempt to discover a colour reaction resulted in the elaboration of the following test for hyponitrites. Addition of resorcinol and potassium periodate to a neutral (or at least not strongly alkaline) solution of a hyponitrite produces an orange- or cherry-red colour which is quite intense after 5 minutes, if the substance is present in measurable quantity: after an hour or so, depending on the amount of hyponitrite present, the solution becomes strongly fluorescent[1]. Should the test solution be strongly alkaline a holly-green colour is produced which becomes red on acidification. This test appears to be sensitive for hyponitrite-nitrogen down to a concentration of only 10 parts per million: it gives reliable results in Beesley's medium and in presence of ammonia, nitrite and nitrate and has proved very valuable in the present investigation.

As far as has been ascertained, the only other substances which behave in a similar manner with resorcinol and potassium periodate are hydroxylamine, borates and bicarbonates. The first-named usually gives a much yellower colour and, moreover, it reduces Fehling's solution whereas hyponitrites do not. Borates and bicarbonates were not present during the current investigation so that no interference was experienced from this source.

It may be mentioned that Blom's test for hydroxylamine, depending on the Griess colour reaction for nitrites after oxidation with iodine, is not specific, being given also by hyponitrites in certain concentrations.

*The catalytic oxidation of nitrous acid to nitric acid.*

It has been established that nitrites in solution decompose according to equation (*a*). Barritt has shown, and the writer has confirmed, that in acid solution in presence of air practically the whole of the nitrite is converted into nitrate. The nitric oxide formed in solution is oxidised to nitrogen peroxide by oxygen from the air and is then converted into nitrous and nitric acids. Thus the reaction is continuous until the whole of the nitrous acid is oxidised according to the equations:

$$(a) \qquad 3HNO_2 = HNO_3 + 2NO + H_2O.$$

$$(b) \quad 2NO + H_2O + O_2 = HNO_2 + HNO_3.$$

$$(c) \qquad 2HNO_2 + O_2 = 2HNO_3.$$

[1] Although satisfactory results were obtained with the culture media employed, it is not known how far the test is of general application. Experiments carried out by Dr W. R. Wooldridge have shown that, if the test solution is buffered at a $p_H$ value between 5 and 6, an old gold colour is developed immediately resorcinol and potassium periodate are added in presence of hyponitrite, while fainter pink colours are produced after an interval of one minute with borates and bicarbonates. The colour developed with hydroxylamine is of a deep reddish hue and not yellow.

The results of a series of experiments showed that the rate of disappearance of nitrite from solution increased with increasing temperature and also with the degree of acidity of the solution. The data indicate that the removal of nitrite from acid soils by chemical means must be rapid, and it seems improbable that *Nitrobacter* occurs in such soils.

*The photochemical oxidation of ammonia to nitrous acid.*

The recent investigations of Dhar and his co-workers [Rao and Dhar, 1931; Dhar *et al.*, 1933] show clearly that nitrite formation can be effected in the absence of micro-organisms: it is pointed out that nitrification in soil is greatest in the top layer where photochemical activity is at a maximum. These workers found that the velocity of the reaction was increased in the presence of certain photosensitisers (of which titania proved to be the most active) but decreased with increasing acidity of the medium. It is submitted that, in tropical countries, the production of nitrite by photochemical agency may be of much greater significance than that resulting from microbiological activity.

In the present research experiments were carried out to determine the extent of the photochemical oxidation of ammonium sulphate, and the results obtained by the Indian investigators were confirmed. Although no attempt was made to follow the course of the reaction closely, it appears that the transformation is largely a surface phenomenon. It was not possible to make the irradiation process a continuous one and this fact must be taken into account in the interpretation of the results. It can be stated, however, that if the reverse change takes place in the dark it does so to an inappreciable extent.

The photochemical oxidation of ammonia to nitrous acid is accompanied by the formation of hyponitrous acid, according to the resorcinol-potassium periodate test, and this substance was detected in greatest amount during the early stages of the experiments but was never present in sufficient concentration to admit of quantitative estimation. In the studies of the biological oxidation of ammonia to nitrous acid, to be described in the next paper, however, the production of hyponitrous acid, as detected by the resorcinol-potassium periodate test, was confirmed by quantitative determinations.

*The photochemical reduction of nitric acid to nitrous acid.*

Although no systematic study of the process of denitrification was made at this stage, a few experiments were made to determine the extent of the reduction of nitrate to nitrite by the action of light. The procedure employed was similar to that used to investigate the photochemical oxidation of ammonia. It was found that the velocity of the photochemical reduction of nitrate was considerably greater than the rate of oxidation of ammonia to nitrite, under identical conditions. As in the case of the oxidation process, the transformation occurred at the surface but, unlike this former change, the reaction proceeded for a short time after removal of the light source. If the reverse action takes place in the dark it must be inappreciable.

## Experimental.

*The catalytic oxidation of nitrous acid to nitric acid.*

In a series of experiments the relation between the rate of disappearance of nitrite from aqueous solutions of potassium nitrite and the $p_H$ value and the temperature was investigated. The solutions were preserved in stoppered flasks

and incubated in the dark: the nitrite was estimated by means of standard potassium permanganate and sodium oxalate solutions, and the $p_H$ was determined by the quinhydrone electrode.

It was found that, in the absence of air sufficient to supply oxygen according to the equations on p. 1577, the oxidation process was brought to a standstill and the nitrite remained in solution although the latter might be very acid.

In Tables I, II and III are given the losses of nitrite from aqueous solutions of potassium nitrite at different $p_H$ values and different temperatures. The results given in Table III are expressed graphically in Fig. 1.

Table I. *Loss of nitrite from aqueous solution at different* $p_H$ *values.*

Initial concentration, 0·222 g. $KNO_2$ per 100 ml. solution. Temperature 21°.

| $p_H$ value of solution | Percentage nitrite loss after 45 days |
|---|---|
| 4·14 | 100·0 |
| 5·08 | 27·1 |
| 5·23 | 18·6 |
| 5·41 | 11·5 |
| 5·67 | 6·4 |
| 6·10 | 1·2 |

Table II. *Loss of nitrite from aqueous solution at different* $p_H$ *values.*

Initial concentration, 0·221 g. $KNO_2$ per 100 ml. solution. Temperature 32°.

| | Percentage nitrite loss | |
|---|---|---|
| $p_H$ value of solution | After 1 day | After 8 days |
| 4·35 | 49·4 | 100·0 |
| 4·42 | 34·7 | 89·5 |
| 4·68 | 15·4 | 54·3 |
| 5·32 | 1·0 | 9·6 |
| 6·93 | 0·0 | 1·0 |
| 8·38 | 0·0 | 0·0 |

Table III. *Loss of nitrite from aqueous solution at different temperatures.*

Initial concentration, 0·239 g. $KNO_2$ per 100 ml. solution. $p_H = 4·0$.

| | Percentage nitrite loss | | |
|---|---|---|---|
| Time in days | 4° | 32° | 37° |
| 1 | 30·6 | 56·2 | 91·6 |
| 2 | 51·7 | 94·2 | 97·7 |
| 3 | 70·2 | 97·5 | — |
| 4·75 | 92·4 | — | — |
| 6·75 | 100·0 | — | — |
| Initial concentration, 0·187 g. $KNO_2$ per 100 ml. solution. $p_H = 4·89$. | | | |
| 1 | 10·8 | 17·8 | — |
| 2 | 10·8 | 18·7 | — |
| 3·25 | 14·2 | 28·9 | — |
| 7 | 18·7 | 34·5 | — |
| 16 | 24·6 | 38·1 | — |
| 24 | 31·2 | 42·7 | — |

In one experiment the nitrite *plus* nitrate-nitrogen remaining in solution was estimated, by reduction with Devarda's alloy, and the following figures were obtained.

| $p_H$ | Initial nitrous N content | Nitrous N content on 45th day | Total nitrous + nitric N on 45th day |
|---|---|---|---|
| 5·23 | 0·0366 | 0·0297 | 0·0355 |
| 5·08 | 0·0366 | 0·0266 | 0·0348 |

*The photochemical oxidation of ammonia to nitrous acid.*

*Experiments with sunlight.* A quantity of Beesley's medium[1] was inoculated with a few drops of inoculum known to contain nitrite-forming organisms, and 10 ml. were measured into a number of test-tubes. Half of the tubes were

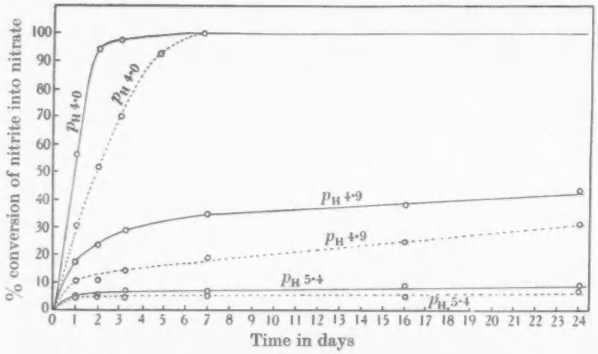



Fig. 1. Full lines, temperature 32°. Broken lines, temperature 4°.

kept in a dark cupboard, and the rest were exposed to sunlight in the window of the laboratory. Judging by the colour developed with the Griess reagent, at first the tubes in the dark cupboard contained more nitrite but after the 53rd day the tubes in the window were richer in nitrite.

The experiment was repeated with sterilised media, but nitrite formation was very slow: after 162 days the nitrite content of the tubes in the window was considerably greater than that of those kept in the cupboard. In neither experiment was nitrite formation accompanied by gas production.

In a further experiment, a 9·2 % solution of ammonium sulphate was prepared and excess of calcium carbonate was added. The solution was preserved in stoppered flasks, one of which was placed in the window of the laboratory whilst the other was kept in a dark cupboard. Qualitative tests showed the presence of hyponitrite and nitrite to a much greater extent in the light than in the dark. The nitrite present was estimated colorimetrically by the Griess-Ilosvay method.

| | mg. nitrous-N per 100 ml. solution | |
|---|---|---|
| | 61 days | 105 days |
| (*a*) Light | 0·013 | 0·190 |
| (*b*) Dark | 0·000 | 0·008 |
| Difference between (*a*) and (*b*) | 0·013 | 0·182 |

*Irradiation by mercury vapour lamp.* In one experiment a 0·2 % solution of ammonium sulphate, in presence of excess of calcium carbonate, was irradiated by means of a 100 volt D.C. mercury arc. The solution was placed under the arc in an open beaker and, at intervals, the nitrite content of the solution was determined colorimetrically. A correction was made for water lost from the solution by evaporation (Table IV).

[1] The composition of Beesley's medium is: 0·95 g. ammonium sulphate, 0·2 g. potassium dihydrogen phosphate, 0·2 g. sodium chloride, 0·066 g. $MgSO_4$, $7H_2O$, 20 g. calcium carbonate and 1000 ml. distilled water.

Table IV. *Nitrite formation from ammonium sulphate by irradiation.*

| Hours of irradiation | mg. nitrous N per 100 ml. solution | Hours of irradiation | mg. nitrous N per 100 ml. solution |
|---|---|---|---|
| 12 | 0·025 | 69 | 0·059 |
| 20 | 0·025 | 74·5 | 0·073 |
| 28·5 | 0·032 | 78 | 0·072 |
| 36·5 | 0·037 | 81·5 | 0·080 |
| 41·5 | 0·036 | 85·5 | 0·078 |
| 50 | 0·040 | 89·5 | 0·081 |
| 58 | 0·050 | 94·3 | 0·081 |
| 61·5 | 0·054 | | |

The experiment was repeated and the nitrite content of the solution was determined at more frequent intervals (Table V).

Table V. *Nitrite formation from ammonium sulphate by irradiation.*

| Hours of irradiation | mg. nitrous N per 100 ml. solution | Hours of irradiation | mg. nitrous N per 100 ml. solution |
|---|---|---|---|
| 1 | 0·004 | 5 | 0·026 |
| 2 | 0·009 | 6 | 0·034 |
| 3·5 | 0·011 | 6·5 | 0·040 |
| 4·5 | 0·022 | | |

The solution was stored in a dark cupboard during the night and a determination of the nitrite content on the following morning showed that no change had taken place.

*The photochemical reduction of nitric acid to nitrous acid.*

*Experiments with sunlight.* A series of test-tube experiments with aqueous potassium nitrate showed that nitrite formation in tubes kept in the laboratory window was considerable compared with that in tubes in a cupboard. No gas evolution occurred during the reduction process.

A 0·25 % solution of potassium nitrate was prepared and placed in stoppered glass flasks, one of which was placed in the window, the other being kept in a cupboard. The nitrite present was estimated colorimetrically: no hyponitrite was detected in solution by the resorcinol-potassium periodate test.

| | mg. nitrous N per 100 ml. solution | |
|---|---|---|
| | 69 days | 114 days |
| Light | 0·003 | 0·024 |
| Dark | 0·000 | 0·000 |

*Irradiation by mercury vapour lamp.* A 0·25 % solution of potassium nitrate, in an open beaker, was irradiated by means of a 100 volt D.C. mercury arc and the nitrite content was determined at intervals by the Griess-Ilosvay colorimetric method (Table VI). A correction was made for the water lost by evaporation.

Table VI. *Nitrite formation from potassium nitrate by irradiation.*

| Hours of irradiation | mg. nitrous N per 100 ml. solution | Hours of irradiation | mg. nitrous N per 100 ml. solution |
|---|---|---|---|
| 1 | 0·004 | 11 | 0·075 |
| 2 | 0·008 | 12 | 0·077 |
| 3 | 0·010 | 13 | 0·080 |
| 4 | 0·020 | 14 | 0·080 |
| 5 | 0·038 | 15 | 0·088 |
| 6 | 0·045 | 16 | 0·094 |
| 7 | 0·053 | 17 | 0·098 |
| 8 | 0·062 | 18 | 0·099 |
| 9 | 0·063 | 19 | 0·109 |
| 10 | 0·074 | | |

SUMMARY.

An investigation of the mechanism of the nitrification of ammonium sulphate has shown that certain of the stages may be effected by microbiological, chemical or photochemical agency.

The change $NH_3 \rightarrow HNO_2$ may occur as a result of bacterial agency or by the action of light and in both cases hyponitrous acid has been detected as an intermediate product. The change $HNO_2 \rightarrow HNO_3$ may be the result of bacterial activity or it may take place chemically, as the reaction is catalysed by the hydrogen ion. At $p_H$ values above 5·0 the loss of nitrite in this way is not appreciable, but at lower values the loss may be considerable. This is of some practical importance and suggests that, under certain conditions, nitrification in tropical soils may be largely a chemical process.

It has also been established that the photochemical change $HNO_3 \rightarrow HNO_2$ is by no means inappreciable.

With resorcinol and potassium periodate, hyponitrites give an orange- or cherry-red colour which is quite intense after 5–10 minutes and becomes strongly fluorescent after standing for an hour or so. Similar colours are given by hydroxylamine, borates and bicarbonates with these reagents but hydroxylamine is readily detected by its reducing action on Fehling's solution.

The author wishes to express his indebtedness to the Research Council of Messrs Imperial Chemical Industries, Ltd., for a grant which rendered this investigation possible, and to Mr H. J. Page and Prof. H. Raistrick for their continued interest.

REFERENCES.

Barritt (1933). *Ann. Appl. Biol.* **20**, 165.
Beesley (1914). *J. Chem. Soc.* **105**, 1014.
Berthelot and Gaudechon (1911). *Compt. Rend. Acad. Sci.* **152**, 522.
Blom (1928). *Biochem. Z.* **24**, 392.
Dhar, Bhattacharya and Biswas (1933). *Soil Sci.* **35**, 281.
—— and Rao (1933). *J. Indian Chem. Soc., Sir P. C. Ray 70th birthday commemoration volume*, 81.
Lindsay and Rhines (1932). *J. Bact.* **24**, 489.
Rao and Dhar (1931). *Soil Sci.* **31**, 379.
Schloesing and Müntz (1877). *Compt. Rend. Acad. Sci.* **84**, 301.
—— —— (1878). *Compt. Rend. Acad. Sci.* **85**, 1018.
—— —— (1879). *Compt. Rend. Acad. Sci.* **86**, 892.

# CCX. SURFACE FILMS OF ERGOSTEROL AND ITS IRRADIATION PRODUCTS.

By JAMES FREDERIC DANIELLI and NEIL KENSINGTON ADAM.

*From the Sir William Ramsay Laboratories of Inorganic and Physical Chemistry, University College, London, and Imperial Chemical Industries, Ltd.*

(*Received June 30th, 1934.*)

This paper describes a continuation of the work of Rosenheim and Adam [1929] on unimolecular films of derivatives of ergosterol, spread on a water surface. Its object is to obtain information as to the properties of the molecules in these films, in the hope of throwing fresh light on the constitution of the various products. While some progress has been made, there are still many points in the interpretation of the curves presented in this paper whose meaning is obscure; it seems desirable at this stage however to record the results obtained.

Surface pressure and surface potential measurements were made; surface pressure according to the technique of Adam and Jessop [1926, 1; *cf.* Adam, 1930]; surface potential with one of the instruments described by Adam and Harding [1932; 1933], the method being essentially the same as that used by Guyot [1924], Frumkin [1925], Schulman and Rideal [1931] and other workers. The completeness of spreading of the films was occasionally verified by examining them with a cardioid dark-ground condenser in the bottom of the trough, using the method of Zocher and Stiebel [1930], as modified by Adam [1933]. The solidity or otherwise of the films was tested by sprinkling a little talc on the surface and noting whether the particles could be blown about freely on the surface, or whether the surface behaved like a solid film.

The films have been investigated on $N/100$ hydrochloric acid and on various strengths of permanganate. The substances investigated were ergosterol, lumisterol, tachysterol, calciferol and two "suprasterols," the last five being formed successively during the irradiation of ergosterol; pyrocalciferol, formed by the action of heat on calciferol; $\alpha$-dihydroergosterol, in which one of the double bonds in the ring system of ergosterol is hydrogenated; $\alpha$- and $\beta$-ergostenols, in which the double bond in the side-chain is hydrogenated in addition to one of those in the ring system; $\beta$- and $\gamma$-ergostadienetriols, formed by the action of perbenzoic acid on ergosterol at low and high temperatures respectively. Our warmest thanks are due to Drs Rosenheim and Callow, of the National Institute for Medical Research, to Prof. Windaus and Dr Lüttringhaus, of Göttingen, and to Mr A. L. Bacharach, of Messrs Glaxo, Ltd., for specimens of these compounds.

In the figures, areas per molecule are plotted horizontally, the scale of surface pressures in dynes per cm. being indicated vertically on the left of the diagrams; on the right, the scale of the surface potentials $\Delta V$ (the change in contact potential between liquid and air caused by the presence of the films) in mv. and the values of $\mu$, in e.s.u. $\times 10^{-21}$ per molecule calculated from Helmholtz' equation

$$\Delta V = 4\pi n\mu,$$

$n$ being the number of film molecules per cm.$^2$ $\mu$ is undoubtedly related to the vertical component of the dipole moment of the molecules in the surface films, but contains also terms of unknown magnitude depending on the degree of re-orientation of the molecules of water near the surface and of re-arrangement of the ions, which occur when the film is put on the surface.

*Films on N/100 hydrochloric acid.*

Fig. 1 shows the results obtained with ergosterol and its various irradiation products. Ergosterol has a smaller area (about 37·5 sq. Å. at no compression) than that given by Rosenheim and Adam [1929] and by Fosbinder [1933], who gave 40·5 sq. Å. within 0·5 sq. Å. as the area at no compression. We now find that if the surface pressure curves are taken rapidly, an area of about 40·5 sq. Å. is obtained, but that the film is exceedingly rigid, and probably consists of islands of rigid film, not quite closely packed. It appears to collapse under moderate pressure until an area of 37·5 sq. Å. is reached, when the film can be

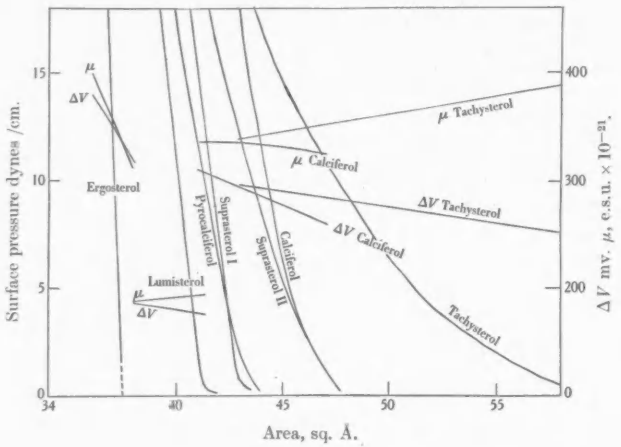



Fig. 1.

compressed and decompressed several times without further apparent collapse. At this stage we think that the rigid islands of film have been so deformed that the space between them is negligible, and the area is nearly that of closely packed molecules. On several occasions a bridge across the full width of the trough (14 cm.) was formed, which resisted a pressure of 4 dynes per cm. on one side only; the thickness of the film is so small that this corresponds to a pressure of the order of 20 atmospheres.

There is the possibility that the film was slightly collapsed at 37·5 sq. Å. Examination with the cardioid dark-ground condenser showed very slight signs of collapse; but forcible reduction of area by a further 10 % caused a very great increase in the amount of visible material expelled from the film (which is itself invisible); we think that the amount of unspread material, if any, in the film at 37·5 sq. Å. is less than would occupy an additional 1 sq. Å. when spread. The larger areas reported previously are probably due to the films being imperfectly packed owing to the rigidity of the islands of film first deposited.

The changes of area with successive irradiation products are very remarkable. The area increases with the progress of irradiation up to tachysterol, and then decreases. Rosenheim and Adam [1929] found a steady increase in area on irradiation, but not this diminution of area in the later stages of irradiation; we think that the substances here used are of much greater purity.

The surface potential became constant, within about 10 mv. over the whole films, when the pressure exceeded about 1·5 dynes. $\mu$ lay between 3 and $4 \times 10^{-19}$ e.s.u. for most of the substances, but was decidedly lower, about $1{\cdot}9 \times 10^{-19}$, for lumisterol. $\mu$ rises considerably when ergosterol is compressed, although the area diminution is small.

Ergosterol and lumisterol formed solid films; the other irradiation products liquid films. α-Dihydroergosterol gave a surface pressure curve almost indistinguishable from that of ergosterol; it is solid and shows a slight tendency to

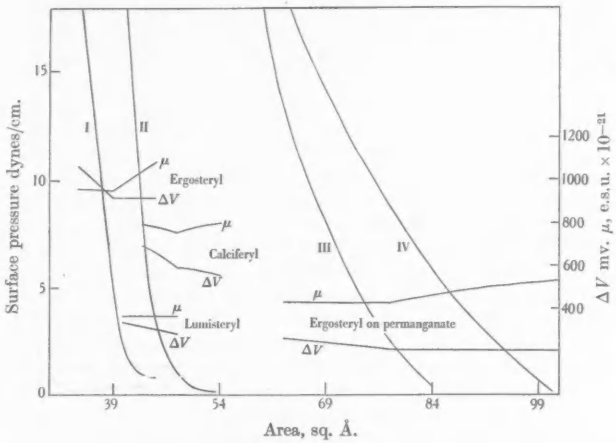



Fig. 2. Dinitrobenzoates.

I. Ergosteryl } on
II. Lumisteryl and calciferyl } HCl.
III. Calciferyl } on per-
IV. Ergosteryl } manganate.

collapse. This substance is believed to have one of the double bonds in the ring system hydrogenated. Two ergostenols, α and β, which have one double bond only in the ring system, gave a rather larger area, approaching 45 sq. Å. at no compression; α-ergostenol films were much more compressible than β, the latter having a compressibility not much larger than that of ergosterol itself. The β-ergostenol was probably a complex mixture.

Fig. 2, curves I and II, III and IV, show the results obtained with the 3:5-dinitrobenzoates of ergosterol, lumisterol and calciferol. In spite of the large size of the dinitrobenzoyl radical, there is not any large increase in the area of the films. Indeed calciferol dinitrobenzoate actually packs into a smaller area than calciferol itself. As however the areas are all over 37 sq. Å., this result is not surprising, for the benzene ring itself is known to have a cross section, packed closely and upright in the films, of only 24 sq. Å., and an additional 12 sq. Å. are likely to be sufficient to accommodate the two nitro-groups. But the results show that the dinitrobenzoate molecules are so orientated on the

surface that the dinitrobenzoyl residue forms approximately a continuation of the general direction of the sterol ring system down into the water.

The esterification with the dinitrobenzoyl group increases the surface potential and $\mu$, but not equally in the three cases. The increase in $\mu$ is much greater in the case of ergosterol than with the other two and is least with lumisterol. A curious feature of the $\Delta V - A$ and $\mu - A$ curves is the abrupt change in direction at a certain stage of the compression, as if some part of the molecule were being re-orientated so as to alter the vertical component of its dipole moment, as the film is compressed to a certain area beyond which the re-orientation with decrease in area either ceases or changes abruptly in direction.

*Oxidation of the films by permanganate solutions.*

It was found by Adam and Jessop [1926, 2] that the oxidation of surface films of unsaturated compounds often causes a drastic change in the orientation of the molecules, through changing the not very water-attractive group, —CH=CH— into —CH(OH).CH(OH)—, which is strongly attracted to the water. In particular, if the double bond is present in a part of the molecule of an aliphatic compound, remote from the polar end group providing the first anchorage, the molecules lie flat on oxidation and the films usually become gaseous instead of coherent. Hughes and Rideal [1933] have shown that the velocity of oxidation depends on the ease of access of the double bonds in the molecule to the surface of the permanganate solution; thus when oleic acid films were compressed so much that the molecules were inclined at a steep angle to the surface, the double bond was oxidised much more slowly than when there was more room on the surface, the motions of the hydrocarbon chains in the film under small compression (the film is of the liquid expanded type) permitting the double bond to come into more frequent contact with the underlying liquid. It was hoped that by careful investigation of the rates of oxidation of compounds of the sterol group, on oxidising solutions of different strengths, information might be gained as to the position and accessibility to the water surface of the double bonds. Other considerations besides simple accessibility to the underlying solution are, however, likely to influence the rate of oxidation; it is well known that conjugated double bonds are more readily oxidised than isolated ones.

Potassium permanganate solutions varying in strength from 2 % downwards were employed. For the surface potential measurements, since a silver-silver chloride electrode was used to make the connection between the solution and the rest of the electrical circuit, the presence of some chloride ion in the solution was desirable, in order to maintain a constant and reproducible potential at this junction. The permanganate solutions were therefore made up with equal weights of potassium chloride and potassium permanganate; the addition did not appear to affect the manner of oxidation of the films.

Strong solutions were usually found rather unsatisfactory, the oxidation proceeding in an uncontrolled manner to a stage at which the films either collapsed or dissolved. Most of the substances discussed in this paper contain two or more double bonds; complete oxidation to the stage where one molecule of hydrogen peroxide is added to each double bond would therefore result in the presence of five or more water-soluble groups in the molecule, a state of affairs which would almost certainly result in dissolution of the film. It was, however, noticed that calciferol was the most readily oxidised to the stage of dissolution, while $\alpha$-dihydroergosterol remained unattacked even on 1 % permanganate

containing $N/10$ sulphuric acid. Cholesterol was oxidised to an initial stage, on this solution, in about 10 minutes. Except where otherwise stated, the permanganate solutions were made up with the addition of potassium chloride but without adding acid.

On more dilute solutions (0·1–0·001 %) it was often possible to recognise a fairly definite end to an initial stage of oxidation, which presumably corresponds to the conversion of one of the double bonds into the dihydroxylated stage. Further changes in the films occurred but were slow, so that the characteristics of the films in this stage of oxidation could be approximately ascertained. Often the same initial stage of oxidation was found over a wide range of dilutions of the permanganate.

The curves of oxidised ergosterol, lumisterol and suprasterol I (curves IV, III and II respectively of Fig. 3) are so nearly the same that the differences

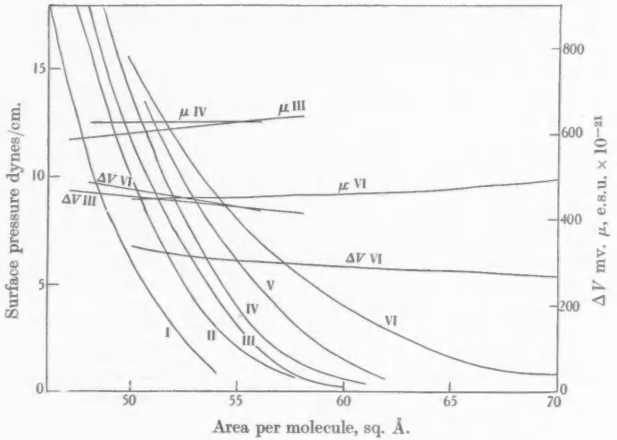



Fig. 3.

I. Suprasterol II. II. Suprasterol I. III. Lumisterol.
IV. Ergosterol. V. Pyrocalciferol. VI. Calciferol.

are probably experimental errors. They end at about 60 sq. Å. at no compression and occupy about 51 at 10 dynes. The oxidised films of calciferol occupy a decidedly larger area (*ca.* 70 sq. Å. at low compressions), those of pyrocalciferol a slightly larger area and of suprasterol II a slightly smaller area. Surface potentials were measured with ergosterol, lumisterol and calciferol; $\mu$ changed very little on compressing the films and was always a good deal higher than with the unoxidised films, especially with lumisterol, where the increase of $\mu$ is approximately threefold on oxidation. The films of ergosterol and lumisterol, after oxidation to this stage, are practically indistinguishable from each other, either in surface pressure or surface potential, although the unoxidised films are very different, especially in surface potential. Tachysterol was not oxidised by 0·1 % permanganate.

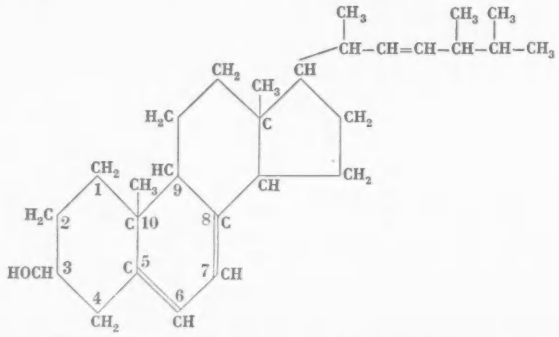
The dinitrobenzoates of ergosterol and calciferol were also examined; they were decidedly more resistant to oxidation than the sterols themselves but were attacked by 0·25 and 0·1 % solutions, on which nearly the same initial stage of oxidation was obtained. On stronger solutions no definite stage of

oxidation could be found; on weaker the rate of oxidation was inconveniently slow. The areas of these oxidised films are very much greater than those of the same substances on non-oxidising solutions. It is probable that the change in orientation of the molecule, caused by the introduction of additional points of anchorage to the water on oxidation at the double bonds, is drastic enough to cause the large dinitrobenzoyl group to spread out along the water surface more or less horizontally, for the areas at no compression reach 100 sq. Å. for ergosteryl dinitrobenzoate and 85 for calciferyl dinitrobenzoate. $\mu$ is diminished on oxidation, a further indication that the orientation of the dinitrobenzoyl group, which provides the greater part of the value of $\mu$ in the unoxidised films and is not likely to be particularly easily oxidised, is radically changed in orientation on oxidation of the rest of the molecule. The slower rate of oxidation of the dinitrobenzoates is presumably due to the obstruction of the large dinitrobenzoyl group.

*Rates of oxidation on permanganate solutions.*

The different substances were found to vary very much in the rate at which the area increased on a given strength of permanganate, *i.e.* in their ease of oxidation. In three cases, cholesterol, pyrocalciferol and lumisterol, the course of the reaction was followed and found to be approximately unimolecular. For the purpose of comparing the rates of oxidation, the times required for the area to increase by one-half of the total increase of area found when the initial stage of oxidation is completed were measured. The figures in Table I are for 0·001 % potassium permanganate under 2 dynes pressure at room temperature. The quantitative aspect of these figures should not be emphasised, but we think that considerable differences in the rate of oxidation, in the order shown above, certainly exist.

Taking the formula of ergosterol provisionally as




[Windaus *et al.*, 1934], and constructing a model with the diameter of the carbon atom 1·5 Å., and that of the hydrogen atom 1·0 Å., the cross section of the molecule placed vertically, with the flexible side-chain above the ring system, is about 37 sq. Å. This is in good agreement with the experimentally observed area of ergosterol; it is the smallest area that can be obtained on the above formula for the ring system of the sterols. Such measurements are only approximate, as it is not possible to estimate how much, if any, interlocking of the projecting hydrogen atoms is permissible between adjacent molecules, or on the other hand, whether repulsive forces keep the molecules further apart in the films than corresponds to these crude models in contact without inter-

locking. In the case of long aliphatic chains, closely packed models of this type give areas some 6·5 sq. Å. smaller than the smallest found in films or crystal packing. For ring structures, *e.g.* long chain derivatives of phenol or of *cyclo*hexanol, the models measured with no allowance for interlocking or for intermolecular repulsive forces keeping the molecules apart gave areas about 3 sq. Å. smaller than those found experimentally in the films. These models probably give a minimum cross section for the molecules, which approaches more nearly to the area occupied in natural packing, the more complex the ring systems. The area of 37 sq. Å. is the smallest measured area found with sterols and probably corresponds to the sterol ring system vertically and closely packed.

Table I. *Times of half oxidation on 0·001 % permanganate.*

| | |
|---|---|
| Pyrocalciferol ... ... ... | 20 seconds |
| Lumisterol ... ... ... ... | 45 ,, |
| Calciferol ... ... ... ... | 200 ,, |
| Suprasterol I ... ... ... | *ca.* 200 ,, |
| Ergosterol ... ... ... ... | 330 ,, |
| Tachysterol, Cholesterol, α-Dihydroergosterol ... ... | Very large |

The models show that only one carbon atom, numbered 3, is at the bottom of the molecule if the molecule is vertical. Any other position but 3 for the hydroxyl would require the molecules to be considerably tilted, unless the hydroxyl group is somewhat removed from the water, which is unlikely, as the hydroxyl group provides the anchorage for the water, and the rest of the molecule has a much smaller attraction for water. Model measurements give an area of about 42 sq. Å. for a sterol ring system with a hydroxyl at 4, tilted so as to permit the hydroxyl to be the lowest point of the molecule. We think that the area to be expected, if the hydroxyl were in position 4, would be considerably larger than that observed; and also, since the molecules would be tilted, lateral compression of the film would probably diminish their tilt, so as to produce a greater compressibility than that observed. The evidence from the surface films appears decidedly unfavourable to the position 4 for the hydroxyl, suggested by Rosenheim and King [1934], following Heilbron *et al.* [1933]. Position 2 is improbable for similar reasons, and position 1 still less probable. In constructing the sterols, Nature appears to prefer that constitution which permits the molecules to stand upright in the interfaces.

It is worth noting also that the areas found for the sterols which give only slightly compressible films, mostly between 37 and 41 sq. Å., are much too small for the older formulae for sterols. These would require at least 50 sq. Å. from measurements on models; the original formulae of Rosenheim and King (with hydroxyl at 3) will fit into 37 sq. Å.

The larger areas of the irradiation products may be due to one or more of three possible causes: (*a*) the stereochemical configuration of the carbon ring system may be changed so that the molecules, packed closely and in a vertical orientation to the water surface, would occupy more space; (*b*) the molecules may become tilted through a change in position of the hydroxyl group, *e.g.* to positions 4, 5 or 6; (*c*) they may tilt because of much smaller changes in the molecule, such as stereochemical alteration in the angle of the hydroxyl group to the ring system (epimerisation). The first type of change (*a*) may occur, but would not be likely, alone, to make more than a few sq. Å. difference to the area; it is probably the cause of the differences in area between ergosterol,

sitosterol, cholesterol and coprosterol; change (*b*), an actual migration of the hydroxyl group, is unlikely on chemical grounds and need not be assumed, because a change of the type (*c*) is already known, in one case, to cause a large change in the tilt of the molecules and in the area and compressibility of the films. Adam and Rosenheim [1929] found that cholestenone gave a film of nearly as large an area as tachysterol, while cholesterol occupies 40·7 sq. Å.

The manner in which a change in the nature of a water-attracting group, or a change in its orientation to the rest of the molecule without change in nature, may alter the orientation of the whole molecule may be as follows. The group is a dipole, and the surface potential measurements show that it is nearly always oriented with the positive end uppermost in the surface. There are probably two antagonistic forces tending to orient these dipoles in different ways on the surface. A single, isolated dipole would probably stand vertically on the surface on account of the symmetry of the field of force of the water molecules. But when a large number of these dipoles are packed close together, as in a surface film, their mutual interaction will tend to make the dipoles lie end to end with their axes parallel to the surface. The Van der Waals attraction between the non-polar parts of the molecules will tend to pack the whole molecules side by side vertically, and thermal agitation tends to destroy any orientation. It is doubtful, however, if thermal agitation is an important factor in the case of these very heavy sterol molecules; evidence has been found in certain cases that change of temperature does not appreciably influence the structure of the films.

A change in tilt without shift in the position of the water-attracting group and without change in its nature, may therefore be achieved by so altering the stereochemical configuration of the skeleton that the angle of tilt of the hydroxyl group to the rest of the molecule is changed. Even without changing the arrangement of the carbon atoms, in a saturated ring a hydroxyl group can alter its angle to the ring by 109·5° by simply changing places with the hydrogen on the same carbon atom. We think it likely that the greater part of the change in the form of the curves observed with the successive irradiation products of ergosterol is due to quite simple changes of this nature in the molecule. The changes in the value of $\mu$ indicate some considerable change in the orientation of the hydroxyl group to the ring system during irradiation. The non-precipitation of the irradiation products by digitonin is usually ascribed to such epimerisation of the hydroxyl group. The changes in the case of oxidation of the films also indicate that the double bonds change in position; in pyrocalciferol and in lumisterol they appear to be much more accessible to the water than in the other compounds. It would be impossible to say at present, however, from the results on surface films, precisely what are the changes in position undergone by the double bonds. Stereochemical changes in ring I may be caused, in part, by movements of the double bonds in ring II.

An interesting, if minor, point is the effect of the dinitrobenzoyl group of the esters on the films. This group does not increase the area of the molecules on dilute hydrochloric acid, but very greatly increases it on permanganate, the initial stage of oxidation. The fairly large dinitrobenzoyl group must therefore continue the general direction of the ring system. This is nearly vertical on the non-oxidising solutions, but at a considerable angle of tilt (though probably not horizontal) when the sterol part of the molecule is tilted on the oxidising solutions. The link between the sterol and the esterifying dinitrobenzoyl group cannot be very flexible, unless the dinitrobenzoyl group is itself oxidised.

SUMMARY.

Surface pressure and surface potential measurements on unimolecular films of ergosterol and its irradiation products, and other derivatives, show considerable changes in tilt of the molecules in the films as irradiation proceeds. It is probable that stereochemical changes occur in the carbon skeleton, resulting in changes in the angle of tilt of the hydroxyl group to the ring system during irradiation. The results also afford evidence that the position of the double bonds changes during irradiation.

The results with ergosterol favour position 3 for the hydroxyl group.

One of us (J. F. D.) thanks the Department of Scientific and Industrial Research, and the Surrey County Council, for personal grants held during the progress of this work; we wish again to thank those who have so kindly supplied the material for this work. Part of the cost of the apparatus was met by a grant from the Royal Society.

REFERENCES.

Adam (1930). The physics and chemistry of surfaces, pp. 37–42.
—— (1933). *Trans. Faraday Soc.* **29**, 91.
—— and Harding (1932). *Proc. Roy. Soc. Lond.* A **138**, 411.
—— —— (1933). *Trans. Faraday Soc.* **29**, 837.
—— and Jessop (1926, 1). *Proc. Roy. Soc. Lond.* A **110**, 423.
—— —— (1926, 2). *Proc. Roy. Soc. Lond.* A **112**, 371.
—— and Rosenheim (1929). *Proc. Roy. Soc. Lond.* A **125**, 25.
Fosbinder (1933). *Proc. Roy. Soc. Lond.* A **139**, 93.
Frumkin (1925). *Z. physikal. Chem.* **116**, 486.
Guyot (1924). *Ann. Physique*, **10**, 506.
Heilbron, Samant and Simpson (1933). *J. Chem. Soc.* 1410.
Hughes and Rideal (1933). *Proc. Roy. Soc. Lond.* A **140**, 253.
Rosenheim and Adam (1929). *Proc. Roy. Soc. Lond.* B **105**, 422.
—— and King (1934). *J. Soc. Chem. Ind.* **53**, 196.
Schulman and Rideal (1931). *Proc. Roy. Soc. Lond.* A **130**, 259.
Windaus, Inhoffen and v. Reichel (1934). *Liebig's Ann.* **510**, 248.
Zocher and Stiebel (1930). *Z. physikal. Chem.* **147** A, 401.

# CCXI. CRYSTALLINE INSULIN.

By DAVID AYLMER SCOTT.

*Connaught Laboratories, University of Toronto.*

(*Received April 28th, 1934.*)

During the past few years the problem of isolating insulin in a crystalline form has been of great interest to the author for several reasons. When large quantities of crystalline material are required for chemical or clinical investigations the present methods of separation leave much to be desired. In the hands of an experienced worker a fair degree of success may be attained. Nevertheless, there would seem to be some unknown condition or conditions which must be fulfilled if a good yield of crystals is to be obtained. Attempts to duplicate successful experiments do not always produce a crystalline product. This has been noted by several workers, who have also drawn attention to the fact that different preparations of crude insulin show a marked variation in the ease with which they may be crystallised. Of further interest is the fact that it is a common belief that the chemistry of insulin is closely related to if not identical with that of proteins, and the results of an investigation concerning the exact conditions under which insulin crystallises would be of value for the isolation of other proteins in a pure form.

Insulin was first isolated in a crystalline form by Abel [1926]. The method utilised as the crystallising medium a solution which was highly buffered and consisted of several substances. In addition to ammonium acetate, it contained large quantities of brucine and pyridine. The isolation of the crystals was attributed to the fact that, by the use of such a complex system of weak bases, the acidity could be adjusted to the isoelectric point of insulin so slowly and so exactly that a supersaturated solution of insulin was obtained. The conditions then seemed to be such that the insulin separated in crystalline form. The problem of crystalline insulin was further investigated by Harington and the author [1929]. Using a buffer solution of ammonium acetate containing saponin, they were able to prepare crystals from amorphous insulin. In microscopic appearance, physiological activity and chemical analysis, these crystals were identical with those described by Abel. In this paper we stated that we were "quite unable to offer any rational explanation of the mechanism of the action of saponin in promoting crystallisation." Nevertheless two factors were apparent. One was the power of saponin to inhibit precipitation and thus permit the preparation of a supersaturated solution of insulin at the isoelectric point. The other was the fact that saponin seemed to sharpen the isoelectric point of foreign proteins in the amorphous insulin and thus permit the removal of many of the impurities at a lower acidity than that at which the insulin crystallised.

Since the publication of the saponin method, I have carried out many experiments on the crystallisation of insulin. In view of the fact that they have a distinct bearing upon the experimental work described later in the present paper, they should be mentioned briefly here. During the summer of 1929, many attempts were made to develop a method superior to the saponin procedure. It was felt that much would be gained if saponin could be replaced by some

pure substance which was highly surface-active. Of the many substances used in these experiments, two were found to be of great assistance in the crystallisation of insulin, namely potassium xanthate and thiocarbanilide. Later work, however, showed that equally satisfactory results could be obtained by using definite quantities of various solvents, *e.g.* acetone, propyl alcohol or butyl alcohol. Since good yields of crystals were obtained even when working with 2-litre quantities of insulin solution, this method was an improvement on the original saponin procedure. However, when it became necessary to use a fresh phosphate buffer solution, it was found that it was no longer possible to obtain crystals. The addition of solvents or other substances previously found to be of value in the formation of crystals, did not overcome the difficulty. The fact that the fresh buffer solution was prepared in the same manner as the one which was found to be satisfactory, suggested that some change had taken place in the solutions after preparation. It seemed possible that this change might consist in the absorption of carbon dioxide from the air or the removal of some inorganic substance from the glass bottle. A great number of experiments were carried out with samples of this fresh buffer to which various amounts of carbon dioxide had been added. However, only negative results were obtained. Experiments were also conducted with this buffer solution to which varying amounts of the common ingredients of glass were added. These substances did not aid in the formation of insulin crystals. Since the distilled water used in the buffer solution which yielded crystals was obtained from a tin-lined still, it was thought that traces of tin might be the essential factor. Accordingly, experiments were conducted with the buffer solution to which various amounts of stannic chloride had been added. This substance also failed to promote crystallisation. However, after storing the buffer solution for about 3 months, that is, until January 1930, it was found that it was then satisfactory for use in the production of crystalline preparations of insulin. Again a fresh phosphate buffer solution was prepared and the same difficulties in crystallisation were encountered.

Since it was impossible to attribute successful crystallisation to any particular constituent of the phosphate buffer, the water or glass, and since it seemed likely that inorganic substances played a definite rôle in the crystallisation of insulin, there was a possibility that pancreas might contain inorganic substances which would assist in the crystallisation of insulin. In searching the literature, it was found that pancreas contains appreciable quantities of zinc [Lutz, 1926], cobalt and nickel [Bertrand, 1926]. To ascertain the effects of these metals upon the formation of insulin crystals, work was initiated in which zinc was added to the crystallising medium. The first experiment was conducted in the following manner. Two phosphate buffer solutions containing about 0·1 % insulin and 10 % acetone were adjusted to $p_H$ 6·2. After standing for 24 hours at room temperature, the precipitates were examined microscopically, and found to be completely amorphous. To one of the beakers, a few granules of zinc were added. They were then allowed to stand for 2 more days and again examined. The beaker to which zinc had been added contained the characteristic rhombohedral insulin crystals, whereas the other precipitate remained completely amorphous. Using a solution of zinc chloride, a great number of experiments were carried out to ascertain the minimum amount of zinc necessary to promote the development of crystals in a phosphate buffer solution containing insulin. This amount was found to be about 2 mg. of zinc for each 100 ml. of phosphate buffer-insulin solution. The crystals obtained in this manner were separated from the solution and re-crystallised. A biological assay showed that they contained 25 I.U. of insulin per mg. Similar experiments were then carried out with

nickel chloride and cobaltic chloride. These substances likewise, in the same concentration as in the zinc experiments, greatly facilitated the formation of crystals. Since iron is grouped with cobalt and nickel in the periodic table, experiments were conducted with various concentrations of ferric chloride. It was found that iron was of no value in the formation of crystals. The effect of other elements in Group II of the periodic table was next determined. It was found that the addition of 2 mg. of cadmium per 100 ml. of buffer greatly facilitated crystallisation. Magnesium, however, had no such effect. During 1931, many similar experiments were conducted using small amounts of the heavy metals. None of these metals was found to aid in the separation of crystals from a phosphate buffer solution containing insulin. Thus it was shown that there were at least four metals, namely, zinc, cadmium, cobalt and nickel, which aided in the preparation of crystals from amorphous insulin. Three of these (Zn, Co and Ni) have been reported to be present in the pancreas. Zinc and cobalt were superior to the others in the promotion of the crystallisation of insulin.

Although the mechanism of the action of these metals in the formation of crystals from solutions of insulin was entirely unknown, there seemed to be three possibilities, *viz.* (1) these elements might form nuclei with the phosphate buffer which initiated the crystallisation of the insulin; (2) these elements might combine with amorphous foreign material and thus leave the insulin free to crystallise; (3) an insulin salt might be formed. In view of the fact that all these metals produce crystals having the same microscopic appearance, it was thought that the second possibility was the most plausible one.

During 1932 little or no work was done on the crystallisation of insulin in these laboratories. In 1933, however, it became necessary to prepare a large quantity of crystalline insulin. There were four methods by which this might be done: (1) the brucine-pyridine procedure, (2) the saponin method, (3) the phosphate buffer method, in which the buffer is stored for 3 months before using, (4) modifications of method (3), using small amounts of either zinc, cadmium, nickel or cobalt, which have already been outlined. On account of the difficulties which might be encountered in removing the metallic elements from the final product if any modification of the fourth method was used, it was decided to prepare the material by means of methods 2 and 3. It seemed particularly advantageous to use method 2, since there were at hand samples of saponin which had been found to be completely satisfactory for the process. After a sufficiently large quantity of crystalline insulin had been obtained, the material was recrystallised from a phosphate buffer solution. The product was thoroughly mixed and samples were removed for biological assay and chemical analysis. The results of assays using 400 mice showed that the crystals contained 25 I.U. per mg. The chemical analysis gave carbon 49·79 %, hydrogen 6·91 %, ash 1·46 %. The values for carbon and hydrogen agree remarkably well with those published by Abel, and by Harington and Scott [1929]. The ash, however, was somewhat higher than the values reported for crystalline insulin by Abel. Unfortunately, no ash values were recorded for the insulin crystals isolated by the saponin method by Harington and Scott. In some unpublished work, I have repeatedly found the ash of crystalline material to be approximately 1 %. The ash value of 1·46 %, while higher than usual, is not surprising when it is remembered that the insulin was recrystallised from a phosphate buffer solution. Thus it would undoubtedly contain a small amount of phosphate. Moreover, since one of the prime objects of this work was to obtain as large a quantity of crystalline material as possible from the insulin at hand, all the solutions were

allowed to stand for one week at 6° before removing the crystals. It is not inconceivable that, in this comparatively long time, some inorganic substances might be precipitated. These would then be included with the crystals and would increase the ash content of the final product. In view of my former findings, namely, that small quantities of zinc, cobalt and nickel facilitate the crystallisation of insulin, the ash was tested for the presence of these metals. It was found to contain phosphate and gave positive tests for iron and zinc. It was free from cobalt and nickel. The amorphous insulin from which the crystals had been prepared, also showed the presence of zinc but no cobalt or nickel. Similarly, the saponin used contained zinc and iron but showed negative tests for cobalt and nickel. Some time ago, Prof. Abel was kind enough to send to these laboratories two crystalline preparations of insulin which he had obtained by his brucine-pyridine method. The first preparation contained 1·03 % ash. This ash, to my surprise, gave a strongly positive test for zinc. The tests for cobalt and nickel were negative. This striking result was confirmed by analysis of a second sample of the same material. The second preparation gave an ash content of 0·6 % and likewise showed a positive test for zinc and negative tests for cobalt and nickel.

Attempts were next made to remove the inorganic substances contained in the crystalline material. Isoelectric precipitation was first tried but this procedure removed little or no inorganic material. Experiments were next conducted with isoelectric precipitation in various concentrations of aqueous acetone solutions. It was found that the ash content could be reduced to about 1·0 % by isoelectric precipitation in an aqueous solution containing about 20 % of acetone. If this product were dissolved in a small volume of 0·1 *N* HCl and the insulin precipitated by the addition of alcohol and ether, a material was obtained which contained 0·25 % ash. A second treatment with acid alcohol and ether yielded a product containing 0·15 % ash. This ash was tested for zinc and the test was still found to be positive. A sample of the material containing 0·15 % ash was assayed on 400 mice. It showed a potency of 24·8 I.U. per mg. Experiments were next carried out in an attempt to crystallise this product. From a freshly prepared phosphate buffer solution no crystals were obtained, whereas from a similar solution containing, in addition to the insulin, small amounts of zinc, cadmium, nickel or cobalt, the characteristic rhombohedral crystals readily formed. Since the last traces of metal apparently could not be removed by treatment with alcohol and ether, attempts were made to do so by electro-dialysis. The material so obtained contained 0·13 % ash and still gave a positive test for zinc. This product was assayed on 300 mice and showed no loss in potency. Attempts were made to crystallise this material. Crystals only formed when zinc, cobalt, nickel or cadmium was added to the phosphate buffer solution.

Thus it has been shown that zinc occurs in the crystalline insulin preparations of Abel, in the crystals obtained by the saponin procedure and also in commercial samples of insulin. In view of this apparent affinity between zinc and insulin, it was decided to prepare a quantity of crystals from a buffered solution of insulin, using the method involving the addition of small amounts of zinc chloride. Further it was intended in these experiments to attempt removal of the zinc by treatment with alcohol and ether followed by electrodialysis, as mentioned above, and to ascertain whether this product still retained its physiological activity and the property to crystallise. Zinc was chosen in preference to cadmium, nickel or cobalt for three reasons. In the first place, it has been my experience that it promotes crystallisation slightly better than the

other elements. Secondly, the solubility of zinc chloride in alcohol and ether is such that it is more readily removed than either cobalt, nickel or cadmium. Further, zinc is a metal normally occurring in commercial preparations of insulin. These experiments are described in detail below.

## Experimental.

A phosphate buffer solution was prepared. This contained 335 g. $Na_2HPO_4$, $12H_2O$ and 55 g. $KH_2PO_4$ in 20 litres of freshly distilled water. The reaction of the solution was $p_H$ 7·15. 250 ml. of this phosphate buffer were measured into a 600 ml. beaker, and to it was added an equal volume of distilled water together with 20 ml. of *N* HCl. The $p_H$ was 2·3. Then 20 ml. of an insulin solution containing 600 mg. of protein were added, followed by 5 ml. of 0·5 % $ZnCl_2$ and 50 ml. of acetone. The solution was adjusted to $p_H$ 6·2 by adding 15 ml. of *N* ammonium hydroxide. (The solution is generally clear. It sometimes happens, however, that if the insulin is very impure, there is a distinct haze at this acidity, and under these conditions it is preferable to adjust the reaction to $p_H$ 6·5 to dissolve the precipitate, and then readjust the solution to $p_H$ 6·2 with *N* HCl.) The inside of the beaker was then rubbed with a glass rod and allowed to stand. In about 20 minutes, crystals began to form, and in one hour the solution became quite opaque. After standing at room temperature for 6 hours, the mixture was placed in the refrigerator for 2 days. Twenty-four experiments were conducted in this manner simultaneously. At the end of 2 days, the supernatant liquids were decanted. The crystals were separated by centrifuging and dried *in vacuo*. The yield from the 24 experiments amounted to 7·5 g. Recrystallisation was then conducted in the following manner. The crystals were added to 150 ml. of water containing sufficient *N* HCl to dissolve most of the material. The insoluble material was removed by filtration. 750 ml. of phosphate buffer solution ($p_H$ 7·15) were diluted with an equal volume of distilled water in a 2 litre beaker. This solution was heated to 55° and to it were added 150 ml. of acetone and 30 ml. of the clear insulin filtrate. Thus 1·5 g. of the material prepared by the first crystallisation were used. The solution was immediately adjusted to $p_H$ 6·0 with *N* HCl and the inside of the beaker rubbed with a glass rod. The solution remained clear until it had cooled to about 40°. Crystals then began to form. A series of five experiments was conducted in this manner. After standing at room temperature for 6 hours, they were placed in a refrigerator. At the end of 2 days, the supernatant liquids were decanted, the precipitates combined and the crystals centrifuged in a 50 ml. centrifuge-tube. The crystals were washed three times with distilled water, using 40 ml. each time. They were then dried *in vacuo* and weighed 5·5 g. After being thoroughly mixed, 4·051 mg. were weighed (sample A) and dissolved in 10 ml. isotonic saline ($p_H$ 2·5). This was further diluted and the physiological activity was determined by the mouse method of assay. The standard insulin solution used for all the biological assays in this work was prepared by dissolving 6·206 mg. of International Standard Insulin powder (8·0 units per mg.) in 107 ml. of isotonic saline ($p_H$ 2·5). Samples of this stock solution were suitably diluted with isotonic saline as required for the assays. As shown by the protocols, the crystals contained 25·6 I.U. of insulin per mg. Chemical analysis showed that the crystals contained: C, 49·30; H, 6·91; N, 14·33; ash, 1·68 %.

A large proportion of the inorganic material was removed from the above recrystallised preparation by the following treatment: 0·5 g. of the crystals was dissolved in a 250 ml. centrifuge-tube containing 100 ml. of water acidified with

1·0 ml. of *N* HCl. After the addition of 20 ml. of acetone, the solution was adjusted to $p_H$ 5·3 with *N* NaOH and placed in the refrigerator overnight. In the morning the mixture was centrifuged and the supernatant liquid discarded. The precipitate was dissolved in 10 ml. of 0·1 *N* HCl and the insulin precipitated immediately by the addition of 100 ml. of absolute ethyl alcohol and 140 ml. of ether. The mixture was placed in the refrigerator. The following morning it was centrifuged and the precipitate redissolved in 10·0 ml. of 0·1 *N* HCl. The insulin was again precipitated by adding alcohol and ether and placed in the refrigerator overnight. The following morning, the precipitate was removed by centrifuging and dried *in vacuo*. Four such experiments were conducted in this manner and the precipitates were thoroughly mixed. 6·805 mg. (sample B) were dissolved in 10 ml. of isotonic saline ($p_H$ 2·5) and after further dilution a biological assay was made. As shown by the protocols, this material contained 26·2 I.U. of insulin per mg. The ash content was 0·12 %, and traces of zinc were still present. The product from the four experiments described above was then used in attempts to crystallise the insulin in the following manner. 100 mg. of this insulin powder were dissolved in 50 ml. of phosphate buffer solution containing 4 ml. of *N* HCl. To this were added 50 ml. of water and 10 ml. of acetone. 10 ml. of this solution were measured into each of a series of ten 15 ml. centrifuge-tubes. The first six tubes of this series were then adjusted with *N*/3 ammonium hydroxide to the following acidities, *viz.* $p_H$ 5·23, 5·45, 5·56, 5·84, 5·95, 6·18. To solution No. 7 in this series, 0·1 ml. of 0·5 % $ZnCl_2$ was added, and to samples 8, 9 and 10 similar quantities of solutions of $CdCl_2$, $NiCl_2$ and $CoCl_2$ were added respectively. These last four insulin solutions, containing the metals, were then adjusted to $p_H$ 6·2. The precipitates so produced were in striking contrast to those formed without the addition of metallic salts. Within an hour, the tubes to which zinc, cobalt or cadmium had been added contained large quantities of crystals entirely free from amorphous material. From the solution to which nickel had been added, a precipitate amorphous in appearance was obtained, but on examination the following morning this was found to be completely crystalline also. Furthermore, regardless of which salt had been added, the crystals all had the same microscopic appearance, namely, the rhombohedral form characteristic of insulin. In those tubes which did not contain these salts, the precipitates were amorphous. From an observation of the amount of precipitate in each of the first six tubes, the isoelectric point of insulin free from metals seems to be about $p_H$ 5·5.

Since the ash of the material purified with alcohol and ether still showed traces of zinc, an attempt was made to remove this metal by electro-dialysis. This was done in the following manner. 1·0 g. of the insulin powder in a litre beaker was dissolved in 500 ml. of distilled water containing 2 ml. of *N* HCl. This solution was then electro-dialysed. For this purpose the type of apparatus of King and Dolan [1934] was used, in which the electrodes are placed in dialysing thimbles, through which water flows continuously. In this experiment, the electrodes were strips of platinum 1 × 2 in. in size. During the experiment distilled water acidified with HCl to $p_H$ 2·5 flowed through the dialysing thimbles. The insulin solution in the beaker was stirred continuously. In spite of these conditions, a precipitate continued to form on the dialysing thimble containing the cathode. This precipitate was constantly scraped off and it redissolved in the insulin solution. Dialysis was continued for one hour using a current of 0·3 ampère. At the end of this time the insulin solution in the beaker was filtered, and *N* NaOH added until the first sign of a turbidity appeared. Further additions of 0·1 ml. of alkali were made at half-hour intervals until a flocculent

precipitate appeared. The beaker was then placed in the refrigerator. The following morning the precipitate was removed by centrifuging and the acidity of the supernatant liquid determined. It was found to be $p_H$ 6·3. The precipitate was dissolved in 20 ml. of 0·1 *N* HCl. 10 ml. quantities of this solution were measured into each of two 250 ml. centrifuge-tubes, and to each were added 100 ml. of absolute ethyl alcohol and 140 ml. of ether. Both tubes were then placed in the refrigerator. The following morning, after centrifuging, the precipitates were dried *in vacuo*. These were intimately mixed and 6·99 mg. (sample C) were dissolved in 50 ml. isotonic saline $p_H$ 2·5. An aliquot was further diluted and assayed biologically. As shown by the protocols, this material contained 23·1 I.U. of insulin, and had an ash content of 0·04 %. The ash gave a faintly positive test for zinc.

In attempts to crystallise the product obtained after dialysis, experiments were conducted in the following manner. 100 mg. were placed in a beaker and to them were added 50 ml. of phosphate buffer solution, 50 ml. of water and 4 ml. of *N* HCl. After the insulin had dissolved, 10 ml. of this solution were measured into each of a series of ten 15 ml. centrifuge-tubes. To each tube, 1·0 ml. of acetone was added. The solutions in the first six tubes of this series were adjusted with 0·25 *N* NaOH to acidities between $p_H$ 5 and 6·5. To solutions 7, 8, 9 and 10, 0·1 ml. of 0·5 % $ZnCl_2$, $NiCl_2$, $CoCl_2$ and $CdCl_2$ was added respectively, and the acidities were adjusted to $p_H$ 6·2. All the tubes in the series were rubbed with a glass rod. Within an hour, a flocculent precipitate formed in the first six tubes. In the solution containing the zinc chloride, crystallisation occurred readily, the precipitate settling to the bottom of the tube. In the experiments using the chlorides of nickel, cadmium and cobalt, precipitates also settled, although less firmly than in the zinc experiment. After standing at room temperature for 2 hours, the tubes were placed in the refrigerator. The following morning, the precipitates which had formed in the presence of the metals presented a striking contrast to those in the first six tubes. Without disturbing the precipitate in any tube of the series, a photograph was taken (Plate X). On microscopic examination, it was found that the precipitates in the tubes containing the metals were completely crystalline, whereas the first six in the series were amorphous. The mixtures were then centrifuged and the acidities of the supernatant liquids were accurately determined and found to be as follows: $p_H$ 5·26, 5·43, 5·64, 5·82, 5·98, 6·18, 6·15, 6·20, 6·24, 6·16. The crystalline material in the last four tubes was separated by centrifuging, washed with distilled water and dried *in vacuo*. Samples of each were then weighed and assayed against the International Standard Insulin. The weights of the various samples were 3·316, 4·901, 3·104 and 2·652 mg. respectively. Each sample was diluted to 50·0 ml. with isotonic saline and aliquots further diluted for assay. These samples are designated in the protocols as D, E, F, G, respectively (tubes 7, 8, 9 and 10).

Since, in the above experiments, and in others like them, it was found that the presence of certain metals was of prime importance for the formation of insulin crystals, attempts were made to determine the possibility of promoting crystallisation by some other means. A series of experiments was carried out in which the acidity of the phosphate buffer-insulin solutions was adjusted with ammonium hydroxide instead of sodium hydroxide. The precipitates were amorphous. Experiments were also conducted with a phosphate buffer solution to which no acetone was added, and again amorphous products were obtained. Similar experiments were carried out with buffer solutions of ammonium acetate and sodium acetate. Although various solutions covering a wide range of acidity

*Protocols of mouse tests.*

| Test No. | International Standard Insulin: Dilution | Amount injected per 20 g. mouse ml. | Convulsion rate | Insulin of unknown potency: Dilution | Amount injected per 20 g. mouse ml. | Convulsion rate | Units per mg. |
|---|---|---|---|---|---|---|---|
| Sample A (insulin crystals by zinc method): | | | | | | | |
| 1 | 1/8 | 0·25 | 12/24 | 1/175 | 0·25 | 13/24 | 26·3 |
| 2 | ,, | ,, | 14/24 | ,, | ,, | 7/24 | 18·5 |
| 3 | ,, | ,, | 12/24 | ,, | ,, | 12/24 | 25·0 |
| 4 | ,, | ,, | 9/24 | ,, | ,, | 11/24 | 27·0 |
| 5 | ,, | ,, | 14/24 | ,, | ,, | 15/24 | 26·0 |
| 6 | ,, | ,, | 20/24 | ,, | ,, | 15/24 | 20·0 |
| 7 | ,, | ,, | 7/24 | ,, | ,, | 9/24 | 27·5 |
| 8 | ,, | ,, | 7/24 | ,, | ,, | 14/24 | 34·3 |
| | | | | | | Mean | 25·6 |
| Sample B (amorphous insulin after ether and alcohol): | | | | | | | |
| 1 | 1/8 | 0·25 | 6/24 | 1/300 | 0·25 | 6/24 | 25·6 |
| 2 | ,, | ,, | 11/24 | ,, | ,, | 15/24 | 30·2 |
| 3 | ,, | ,, | 12/24 | ,, | ,, | 15/24 | 28·9 |
| 4 | ,, | ,, | 17/24 | ,, | ,, | 13/24 | 22·0 |
| 5 | ,, | ,, | 15/24 | ,, | ,, | 10/24 | 20·9 |
| 6 | ,, | ,, | 15/24 | ,, | ,, | 16/24 | 26·8 |
| 7 | ,, | ,, | 17/24 | ,, | ,, | 20/24 | 29·6 |
| 8 | ,, | ,, | 16/24 | ,, | ,, | 15/24 | 24·5 |
| | | | | | | Mean | 26·1 |
| Sample C (amorphous insulin after electro-dialysis): | | | | | | | |
| 1 | 1/8 | 0·25 | 13/24 | 1/57 | 0·25 | 16/24 | 26·7 |
| 2 | ,, | ,, | 10/24 | ,, | ,, | 5/24 | 18·2 |
| 3 | ,, | ,, | 10/24 | 1/60 | ,, | 12/24 | 27·1 |
| 4 | ,, | ,, | 8/24 | ,, | ,, | 7/24 | 23·6 |
| 5 | ,, | ,, | 7/24 | ,, | ,, | 5/24 | 22·4 |
| 6 | ,, | ,, | 10/24 | ,, | ,, | 9/24 | 23·9 |
| 7 | ,, | ,, | 13/24 | ,, | ,, | 7/24 | 18·9 |
| 8 | ,, | ,, | 10/24 | ,, | ,, | 9/24 | 23·9 |
| | | | | | | Mean | 23·1 |
| Sample D (recrystallised insulin by zinc method): | | | | | | | |
| 1 | 1/9 | 0·25 | 25/36 | 1/32 | 0·25 | 22/36 | 22·9 |
| 2 | ,, | ,, | 24/36 | ,, | ,, | 22/36 | 23·6 |
| 3 | ,, | ,, | 13/36 | ,, | ,, | 9/36 | 21·7 |
| 4 | ,, | ,, | 16/36 | ,, | ,, | 13/36 | 23·1 |
| | | | | | | Mean | 22·8 |
| Sample E (recrystallised insulin by nickel method): | | | | | | | |
| 1 | 1/9 | 0·25 | 24/36 | 1/50 | 0·25 | 19/36 | 23·1 |
| 2 | ,, | ,, | 15/36 | ,, | ,, | 13/36 | 25·0 |
| 3 | ,, | ,, | 21/36 | ,, | ,, | 20/36 | 25·8 |
| 4 | ,, | ,, | 17/36 | ,, | ,, | 17/36 | 26·3 |
| | | | | | | Mean | 25·1 |
| Sample F (recrystallised insulin by cobalt method): | | | | | | | |
| 1 | 1/9 | 0·25 | 19/36 | 1/30 | 0·25 | 25/36 | 25·3 |
| 2 | ,, | ,, | 20/36 | ,, | ,, | 32/36 | 31·3 |
| 3 | ,, | ,, | 25/36 | 1/35 | ,, | 25/36 | 29·0 |
| 4 | ,, | ,, | 15/36 | ,, | ,, | 16/36 | 29·9 |
| | | | | | | Mean | 28·9 |
| Sample G (recrystallised insulin by cadmium method): | | | | | | | |
| 1 | 1/8 | 0·25 | 17/24 | 1/23 | 0·25 | 14/24 | 22·4 |
| 2 | ,, | ,, | 23/24 | ,, | ,, | 23/24 | 25·2 |
| 3 | 1/9 | ,, | 12/24 | 1/26 | ,, | 8/24 | 21·3 |
| 4 | ,, | ,, | 14/24 | 1/23 | ,, | 19/24 | 28·0 |
| | | | | | | Mean | 24·2 |

were used, only amorphous precipitates were obtained. Only by adding small amounts of one of the four metals, namely, zinc, nickel, cobalt or cadmium, have we been able to crystallise this material, although it has approximately the same physiological activity as crystalline insulin.

## Discussion.

In the present paper, a very brief review of some work on the crystallisation of insulin has been reported. It was noted during 1930 and 1931 that small amounts of various salts (the chlorides of cobalt, cadmium, nickel and zinc) aided in the formation of crystals from amorphous insulin. It was thought at that time, since the same type of crystal was formed irrespective of the salt which was added, that these metals combined with the foreign proteins in the crude insulin preparations, leaving the insulin free to crystallise. This view was questioned, however, when it was found that crystalline insulin, prepared by the phosphate buffer method and the saponin method, contained zinc. When the ash content was reduced to 0·15 %, the insulin, although having the potency of crystalline material, failed to crystallise from a phosphate buffer solution. Crystals, however, could be formed readily when traces of zinc, cadmium, nickel or cobalt were added to the crystallising medium. Furthermore, two samples of crystalline insulin prepared by Prof. Abel by the pyridine-brucine method were found to contain zinc. There is also presented in detail a method for preparing quantities of crystalline material from amorphous insulin by adding small amounts of zinc chloride to a phosphate buffer solution of insulin. These crystals had the same physiological activity and chemical composition as the crystals prepared by other methods. The inorganic material was partially removed from the crystals by isoelectric precipitation, in 20 % aqueous acetone, and by two precipitations with acid alcohol and ether. The product contained 26·2 I.U. of insulin per mg. It contained 0·12 % ash and showed traces of zinc. This material, however, would only crystallise in the presence of zinc, nickel, cobalt or cadmium. A material having an ash content of 0·04 % and containing 23·1 I.U. of insulin per mg. was obtained by electro-dialysis. This material could only be crystallised in the presence of traces of zinc, cadmium, nickel or cobalt.

The fact that the product after electro-dialysis, containing only 0·04 % ash and giving a faintly positive test for zinc, showed certain properties not usually associated with insulin, is worthy of consideration. When acid solutions of this material were neutralised slowly with sodium hydroxide, no flocculation occurred until the solution had an acidity of $p_H$ 6·3—a significant difference from the commonly accepted isoelectric point of insulin. Biological assays showed that the precipitate was slightly less active than crystalline insulin. This was surprising, since in the electro-dialysis experiment, reported in the introductory part of this paper, no loss in potency could be detected. The differences between these two products may be due to the fact that, in the first experiment, dialysis was continued for $\frac{1}{2}$ hour only. The material so obtained, precipitated in a more acid solution, had an ash content of 0·13 %, and gave a positive test for zinc. The discrepancies between these two experiments will be further investigated.

Perhaps, in view of the present findings, the brucine-pyridine method and the saponin method of crystallising insulin should be discussed further. The success of the saponin method can be explained readily by the fact that saponin suitable for crystallising insulin contains appreciable amounts of zinc and is highly surface-active. This latter factor is comparatively important, as very few insulin crystals are obtained when using the zinc method described in this

paper, unless acetone or some other substance which reduces surface tension is used. It is highly probable that the saponin used by Harington and Scott contained zinc or some other inorganic elements essential for the success of their process. Various saponins, upon analysis, were shown by them to contain "large amounts of inorganic matter even including traces of the heavy metals." Several samples of saponin in my laboratory when tested for zinc, all gave positive tests for this element. Further, a sample of saponin which had been found to be very satisfactory for the crystallisation of insulin, contained appreciable amounts of zinc. This fact, in view of the results of the present investigation, may explain in part the success of the saponin method. With respect to crystals prepared by the brucine-pyridine method of Abel, it might be well again to point out that two samples of crystalline insulin from his laboratory contained zinc. The most probable source of the metal is the pancreatic glands from which amorphous insulin is obtained. Lutz [1926] has reported that the pancreas of various species contains appreciable quantities of this element. Working with the pancreatic glands of the ox, Delezenne [1919] found 23 mg. of zinc per kg. of tissue. In the commercial preparation of insulin, the hormone is extracted from the pancreas with acid alcohol. The alcohol is commonly acidified with hydrochloric acid. Thus the solvent which is satisfactory for the extraction of insulin is also one which would dissolve the zinc contained in the pancreatic glands. Perhaps the most important step in the purification of insulin is isoelectric precipitation. Experiments have shown that zinc adheres to insulin protein when precipitated at the isoelectric point. It is thus most likely that all insulin preparations contain zinc. Certainly all the preparations which have been thus far tested gave a positive test for this element. However, in view of the use of slightly different methods of preparation, it is conceivable that all commercial preparations of insulin do not contain the same amount of zinc. This may account for the fact that insulin from one manufacturer sometimes crystallises more readily than that from another source. In the Abel method of crystallisation there is, of course, the added possibility that the brucine contains small amounts of this element. That a phosphate buffer solution, aged for 3–4 months, more readily promotes the crystallisation of insulin can hardly be attributed to small amounts of zinc, nickel, cobalt or cadmium absorbed from the glass, since glass contains little, if any, of these substances. It is possible, however, that some other element in glass is responsible for this phenomenon. It is also possible that traces of other inorganic substances in the glass have dissolved and are combining with the foreign proteins in insulin and thus leaving the zinc in commercial insulin free to initiate crystallisation.

The observation that crystalline insulin prepared by the brucine-pyridine method of Abel or by the saponin method of Harington and Scott contains zinc may explain the fact that solutions of crystalline insulin are slightly less stable than amorphous preparations. In the crystalline material there is a higher concentration of zinc per unit of physiological activity than in amorphous insulin. In solution, this zinc may exhibit reducing properties and thus destroy the physiological activity of the hormone. From this investigation, there arises the question of the possibility of the rhombohedral crystals being a metallic salt of insulin. There are at least two reasons for believing that such may be the case. In the first place, the addition of small amounts of zinc, cadmium, nickel or cobalt to solutions of amorphous insulin greatly facilitates the formation of crystals. Secondly, if steps are taken to remove the metals from crystalline preparations of insulin, the resulting material cannot be crystallised unless one of the metals cadmium, zinc, nickel or cobalt is added. Thus it

seems possible that a salt of insulin has been formed—probably a simple salt. The fact that the crystals prepared by the addition of the different salts have the same microscopic appearance might lend support to this view. If these metals form simple salts with insulin, other proteins might be expected to behave in a similar manner. Investigations are now being conducted along this line of endeavour. On the other hand, these crystalline preparations of insulin may be double or triple salts. In this connection, one is reminded of the fact that many diabetics who are hypersensitive to commercial preparations of insulin are also hypersensitive to crystalline insulin. If crystalline insulin is the same chemical entity as occurs in human pancreas, the above reactions would not be expected to take place. That crystalline insulin does not give an X-ray pattern under ordinary conditions, is another fact indicating that the crystals may not be a simple salt. Thus it is felt that investigations on the problem of the crystallisation of insulin, together with that of the chemical constitution of the crystals, must be continued, bearing in mind the very definite rôle played by some of the metallic elements.

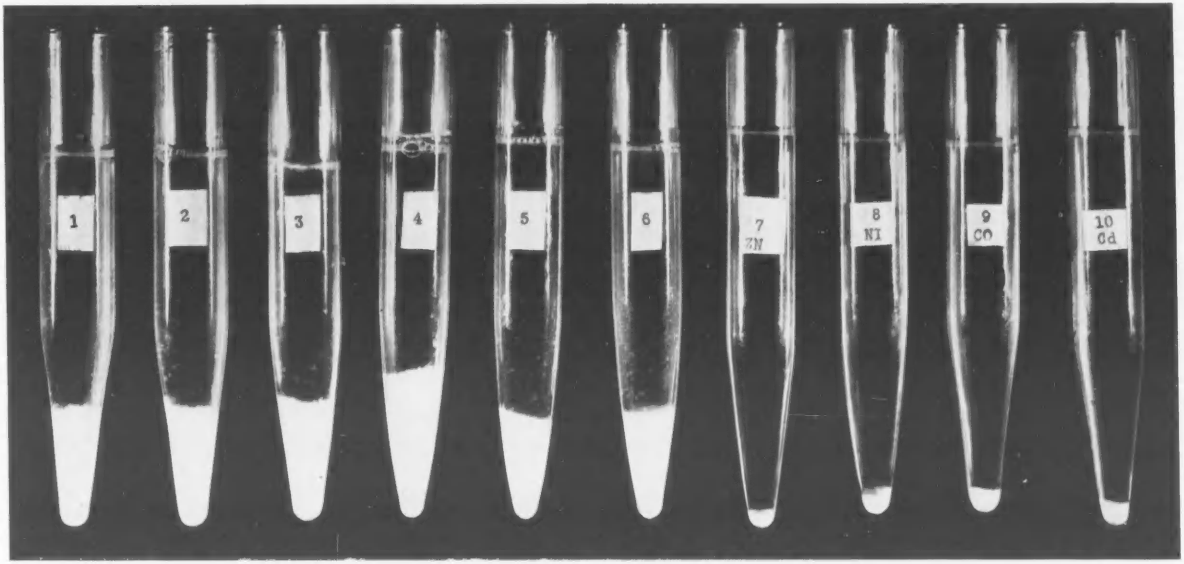
SUMMARY.

It has been shown that insulin crystals, prepared by the pyridine-brucine method or by the saponin method, contain zinc. A method for obtaining insulin crystals from amorphous preparations by means of zinc chloride is described. A process for reducing the inorganic substances contained in the insulin preparation to as low as 0·04 % is given. Such material could not be crystallised by the ordinary procedures. Crystals were obtained only in the presence of small amounts of zinc, nickel, cobalt or cadmium. The significance of these findings has been discussed.

I wish to express my indebtedness to Dr H. Stantial, of the Department of Chemistry, for making the many ash estimations and qualitative tests for inorganic substances. I am also grateful to Dr J. C. FitzGerald and Dr C. H. Best for their interest in this work.

REFERENCES.

Abel (1926). *Proc. Nat. Acad. Sci.* **12**, 132.
Bertrand (1926). *Science*, **64**, 629.
Delezenne (1919). *Ann. Inst. Pasteur*, **33**, 68.
Harington and Scott (1929). *Biochem. J.* **23**, 384.
King and Dolan (1934). *Canad. Chem. Metal.* **18**, 7.
Lutz (1926). *J. Ind. Hygiene*, **8**, 177.




Attempts to recrystallise insulin of low ash content.

Tubes 1–6 contain insulin precipitated from phosphate buffer solutions adjusted to various acidities. All precipitates were amorphous.
In tubes 7–10 are precipitates prepared from buffered solutions of insulin containing traces of the metals as indicated. The solutions were adjusted to the same acidity as the solution in tube 6. These precipitates were crystalline.

MIOM

# CCXII. THE APPLICATION OF THE KOBER TEST TO THE QUANTITATIVE ESTIMATION OF OESTRONE AND OESTRIOL[1] IN HUMAN PREGNANCY URINE.

By SAUL LOUIS COHEN and GUY FREDERIC MARRIAN.

*From the Department of Biochemistry, The University of Toronto.*

(*Received June 23rd, 1934.*)

It is well known that both oestrone and oestriol when warmed with concentrated sulphuric acid give a green-fluorescing orange-coloured solution, thus resembling sterols and the bile acids. The colours given by the latter compounds are discharged by dilution with water, but as Kober [1931] showed, the oestrin colour on dilution with water is changed to a clear red, the green fluorescence being retained. His evidence suggested that this test was highly specific for oestrin. Kober also showed that the addition of phenol to the sulphuric acid eliminated the green fluorescence from, and intensified the red colour of, the finally diluted solution. He stated that under the conditions of his procedure, the intensity of the red colour bore a direct but not linear relationship to the amount of oestrin, and he claimed that by the use of this test it was possible to assay the oestrin content of impure extracts.

The obvious desirability of a rapid chemical method for the quantitative determination of the oestrin content of human urines led the authors to investigate the value of the Kober test for such a purpose.

## Experimental.

### *Modifications introduced into the Kober technique.*

Kober suggested the following technique. About 5$\gamma$ of crystalline hormone (presumably oestrone) are heated to 100° for 2 minutes with 0·2 ml. of a mixture of equal parts[2] of concentrated sulphuric acid and phenolsulphonic acid (*o*-+*p*-). The mixture is then cooled, and after the addition of 0·2 ml. of water is heated momentarily to boiling. Finally the solution is made up to 1 ml. with water and immediately cooled. After standing in the cold for 5–10 minutes, the colour is compared with that of a standard solution of acid cresol red (10 mg. in 100 ml.) in a colorimeter. Kober states that 5$\gamma$ of the hormone give a colour of the same intensity as that of 25$\gamma$ of cresol red.

Since the greater part of the oestrogenic material in human pregnancy urine is oestriol, most of the earlier experiments described here were carried out on

[1] The names *oestrone* and *oestriol* are here used to designate the two oestrogenic substances present in human pregnancy urine which have been known previously as *ketohydroxyoestrin* and *trihydroxyoestrin*, *theelin* and *theelol*, *etc.* This new system of nomenclature has been approved by the majority of the interested English workers [*Nature*, 1933, **132**, 205]. The name *oestrin* is used where it is desired to refer to the oestrogenic material in urine without specifying its exact chemical nature.

[2] It is assumed here that Kober used equimolecular parts of sulphuric and phenolsulphonic acids.

that compound rather than on oestrone. When attempts were made to repeat Kober's technique with pure oestriol, it was not found possible to obtain a colour of the same order of intensity as that reported by him. Furthermore it was found to be impossible to obtain on final dilution solutions of sufficiently uniform tint to enable a single cresol-red solution of any concentration or $p_H$ to be used as a permanent standard of comparison.

In attempting to overcome these difficulties, certain modifications in the conditions of heating were introduced in order to facilitate a rigid standardisation of technique. Even after the introduction of these modifications, slight variations in the tint of the final solution still occurred. It became necessary therefore to use an instrument for colour measurement in which the colour could be analysed and the intensities of its components separately determined. The Lovibond tintometer proved to be suitable for the purpose.

The modified technique used in the initial stages of this work was as follows: 1 ml. of the reagent was measured[1] into an ordinary test-tube which contained from 0·01 to 0·20 mg. of pure dry oestrone or oestriol. The tube was heated in a boiling water-bath for exactly 10 minutes and then immediately cooled in a freezing mixture. The mixture was transferred with 5 % sulphuric acid[2] to a graduated test-tube of about 1 cm. diameter and the volume made up to exactly 4 ml.

The colour of a solution prepared in this way was invariably a yellow-orange, provided that during the dilution the tube was kept at as low a temperature as possible. On heating the solution in a boiling water-bath, the intensity of the yellow component of the colour rapidly decreased, while that of the red component increased, reaching a maximum at about 2 minutes, and then fell slowly. It was found possible to follow these colour changes quantitatively, by removing the diluted solution from the water-bath at 30 second intervals, cooling in a freezing mixture to "fix" the colour and then matching in the tintometer after transference to the standard cell of that instrument.

Colour development curves for oestriol obtained by this procedure are shown in Fig. 1. Similar curves were also obtained with oestrone. It will be seen that the exact time required for the red component of the colour to reach a maximum and for the yellow component to reach a minimum varied with the quantity of oestriol used in the experiment. It was therefore impossible to be sure of observing the "red maximum" of an unknown amount of oestriol by merely heating the diluted solution in a water-bath for a definite time. It was clearly necessary to construct a full colour development curve in every case in order to be certain of recording the true maximum red colour.

*Preparation of reagents and the effect of composition of the reagent on the intensity of the colour reaction.*

The reagents used in these experiments were prepared by measuring out the required amounts of pure concentrated sulphuric acid and of freshly redistilled phenol (warmed to 60°), and mixing thoroughly in a dry, well-stoppered bottle. Owing to the high viscosities of the acid and phenol, prolonged drainage of the pipettes was essential to ensure reasonably accurate measurement.

[1] An ordinary graduated 1 ml. pipette was used. Owing to the high viscosity of the reagent considerably less than 1 ml. was actually delivered. By adopting standard conditions of drainage, the amounts delivered were sufficiently constant to enable reproducible results to be obtained.

[2] Water would be as efficient a diluent as 5 % sulphuric acid for pure oestrone or oestriol, but the latter was found to have certain advantages when colour reactions were carried out on the urine concentrates which were being obtained at this stage of the work.

Since there was some uncertainty in the minds of the authors as to the exact composition of Kober's reagent, an investigation of the effect of varying the

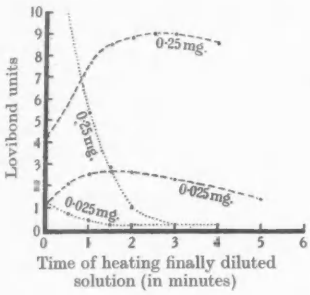



Fig. 1.

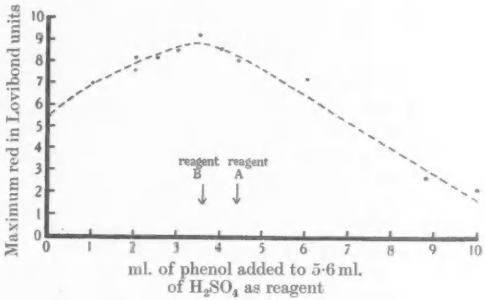



Fig. 2.

Fig. 1. Colour development curves of oestriol treated with 1 ml. of reagent B.
●—–● Red. ●·····● Yellow.

Fig. 2. The effects of variations in the composition of the reagent on the intensity of the colour reaction. 0·2 mg. oestriol, 1 ml. reagent.

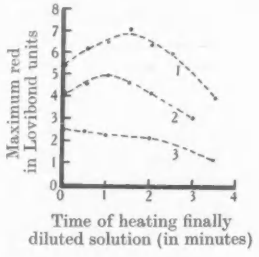



Fig. 3.

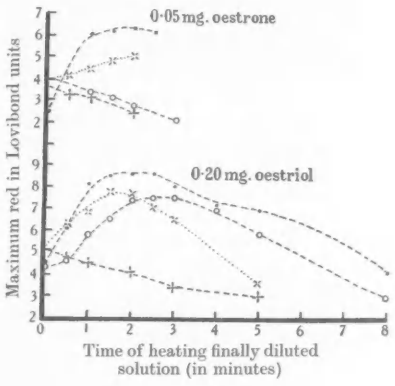



Fig. 4.

Fig. 3. The effect of the addition of water to the reagent upon the colour development curve of oestriol. 0·20 mg. oestriol, 1 ml. reagent A. 1, No added water; 2, 0·05 ml. added water; 3, 0·10 ml. added water.

Fig. 4. The effects of the addition of cholesterol and pregnandiol on the colour development curves of oestrone and oestriol. 1 ml. of reagent B.

●———● Oestrin alone.
×·····× ,, +0·2 mg. pregnandiol.
o———o ,, +0·2 mg. cholesterol.
×———× ,, +0·2 mg. cholesterol + 0·2 mg. pregnandiol.

proportions of phenol and sulphuric acid in the reagent upon the intensity of the colour reaction was carried out. The results of this experiment are shown in Fig. 2. It will be seen that a reagent containing from 3 to 4 parts by volume

of phenol to 5·6 parts by volume of sulphuric acid, gave colours of greater intensity than those given by reagents containing any other proportions of the two components. In some of the experiments described in this paper a reagent was used containing 4·4 parts of phenol to 5·6 parts of acid, which was believed to be identical with Kober's reagent. This will be referred to as "reagent A." In later work a reagent containing 3·6 parts of phenol to 5·6 parts of acid ("reagent B") was used.

At first some difficulty was experienced in preparing different batches of reagent which would produce the same intensities of colour with given amounts of oestrin. It was suspected that these variations might be due to the accidental introduction of traces of water into the reagent. The effect of added water on the course of the colour reaction was therefore quantitatively examined. Small amounts of water were added to the oestrin-reagent mixture before the initial period of heating. As is clear from the results shown in Fig. 3, the addition of water very considerably decreased the intensity of the colour yielded by a given quantity of oestriol. The necessity of keeping the reagent in a tightly stoppered bottle and of ensuring the complete dryness of all pipettes and test-tubes is therefore apparent.

*Colour development curves of oestrone and oestriol in the presence of pregnandiol and cholesterol.*

Partially purified oestrin concentrates were prepared by extracting acidified pregnancy urine with ether, washing the extract with aqueous sodium carbonate and evaporating. Such concentrates when treated with the reagent gave colour development curves which were quite different from those given by pure oestrone or oestriol. The initial colour was red rather than orange, while on heating, the red component failed to rise to a maximum but immediately fell from its initial value. It was evident that these urine concentrates contained substances which were inhibiting the production of the full oestrin colour and which were accelerating its normal rate of fading.

Urine concentrates prepared in the above manner contain not only the total ether-soluble phenolic fraction of the urine, but also all ether-soluble neutral substances. It was therefore decided to investigate the effects of cholesterol and pregnandiol on the oestrin colour development curve, these being the two principal ether-soluble neutral substances which are so far known to occur in human pregnancy urine. Amounts of each and of both were added to oestriol and to oestrone in approximately the same proportions as are known to be present in urine. Either compound separately considerably decreased the intensity of the red colour yielded by given quantities of oestrone or oestriol. When both substances were added together, the inhibitory effect was greatly enhanced. These results, which are shown in Fig. 4, emphasise the necessity for a separation of the phenolic substances, including the oestrone and oestriol, from the neutral fraction before carrying out the colour test. As will be shown in a later section however cholesterol and pregnandiol were not the only colour-inhibitors which must have been present in these crude urine concentrates.

*The separation of oestrone and oestriol from the neutral fraction.*

It is a matter of some difficulty to effect the quantitative separation of the total oestrogenic material in urine concentrates from the neutral fraction. Oestriol may be readily extracted from an ethereal solution with dilute aqueous

alkali hydroxides, but this procedure is not so effective in the case of oestrone, owing to the very feebly phenolic character of the latter compound. A method of separating the oestrone quantitatively from the neutral fraction had therefore to be devised. As a preliminary to the elaboration of such a method the distribution of oestrone between different strengths of aqueous alkali hydroxides and various immiscible organic solvents was studied.

Solutions containing weighed amounts of oestrone in different organic solvents were made up, and aliquot portions of these were shaken with equal volumes of aqueous solutions of sodium and potassium hydroxides of different strengths. After complete separation of the phases, aliquot portions of the organic solvent layers were measured off and evaporated to dryness. Colour reactions were then carried out on the residues so obtained in the manner previously described. By reference to an oestrone standardisation curve constructed specially for the purpose, the amounts of oestrone remaining in the organic solvent after equilibration with the alkali were determined. The results obtained with toluene and ethereal solutions of oestrone equilibrated with solutions of *N*/10 and *N* sodium hydroxide are shown in Table I.

Table I. *Distribution of oestrone between ether or toluene and aqueous sodium hydroxide solutions.*

| Organic solvent | Concentration of NaOH | mg. of oestrone in 10 ml. of organic solvent | | Oestrone in NaOH / Oestrone in organic solvent |
|---|---|---|---|---|
| | | Before equilibration | After equilibration | |
| Ether | *N*/10 | 0·49 | 0·39 | Approx. 1/4 |
| „ | *N* | 0·49 | 0·17 | „ 2/1 |
| Toluene | *N*/10 | 0·47 | 0·28 | „ 2/3 |
| „ | *N* | 0·47 | 0·10 | „ 4/1 |

These results indicated that whereas *N*/10 sodium hydroxide should only slowly extract oestrone from ethereal solution, *N* sodium hydroxide should effect complete extraction from toluene with ease. This suggested that not only would it be possible to separate all the oestrogenic material from the neutral fraction of urine concentrates, but that a quantitative separation of the oestrone from the oestriol fractions might at the same time be effected. The suggested scheme for such a separation is shown in Table II.

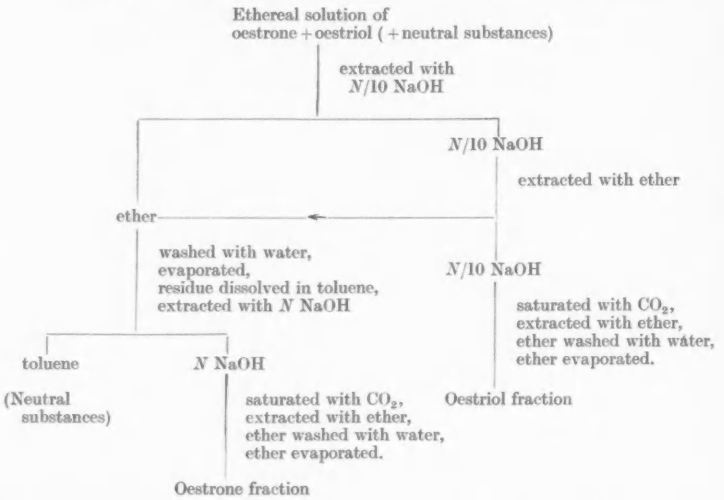
Preliminary experiments on ethereal solutions containing known amounts of oestrone and oestriol indicated that a nearly quantitative separation could be effected by this process. Since these experiments had later to be repeated using the exact technique finally adopted for the urine concentrates (see p. 1611), the preliminary results are not reported.

*The estimation of oestrone and oestriol in pregnancy urine.*

(*a*) *The preparation of oestrone and oestriol fractions from pregnancy urine.* Acid-hydrolysed urine was extracted with ether, the extract was freed from acidic substances by washing with aqueous sodium carbonate and subjected to the further concentration and separation of the phenolic fractions as outlined in Table II.

(*b*) *Further modifications in the colour reaction.* As a result of the discovery that pregnandiol and cholesterol inhibited the development of the maximum red colour produced by oestrone and oestriol, the hope was entertained that the two separated phenolic fractions would give colour development curves

Table II. *Method of separating oestrone and oestriol mixtures.*




similar to those given by the pure substances. This however did not prove to be the case. The colour development curves of the separated phenolic residues still failed to show an increase of the red value up to a maximum, indicating that inhibiting substances persisted.

At first it was feared that this might necessitate the abandonment of the method entirely, since further purification of the phenolic fractions without loss of oestrone and oestriol seemed to be difficult. Fortunately it was found that the substitution of 2 ml. of reagent for 1 ml. in the colour reaction, led to the production of nearly normal colour development curves from these phenolic urine concentrates. The higher concentration of reagent in the finally diluted solution appeared to overcome to a large extent the effect of the colour-inhibitors

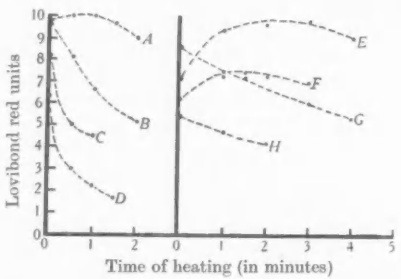



Fig. 5. Colour development curves of oestrone and oestriol fractions of human pregnancy urine. The effects of 1 ml. and 2 ml. of reagent, and of 5 % $H_2SO_4$ and water as diluents.

*A*, oestriol fraction, 2 ml. reagent, 5 % $H_2SO_4$.
*B*, " " " " water.
*C*, " " 1 ml. " 5 % $H_2SO_4$.
*D*, " " " " water.
*E*, oestriol fraction, 2 ml. reagent, 5 % $H_2SO_4$.
*F*, oestrone " " " " "
*G*, oestriol " 1 ml. " " "
*H*, oestrone " " " " "

still present. When water instead of 5 % sulphuric acid was used to effect the final dilution, colour development curves less like the normal resulted. These effects are clearly illustrated in Fig. 5.

Kober observed that the red colour in his test was rapidly and completely destroyed by the addition of hydrogen peroxide. This has been confirmed and the fact has been utilised to eliminate errors due to the presence of "non-oestrin" colour in the finally diluted colour mixture. On adding to this colour mixture a few drops of hydrogen peroxide and heating to 100° for a few minutes, the red colour rapidly disappeared and a pale brown solution usually resulted. (From experiments on pure oestrone and oestriol it was found that heating with peroxide for 2 minutes was sufficient to discharge the colour completely.) The colour of this solution could then be analysed by means of the tintometer and the value of the red component subtracted from the value of the original maximum red. This correction has been applied to all the colour reactions carried out on urine concentrates.

(*c*) *Standardisation curves for oestrone and oestriol.* Before the red values obtained on urine concentrates could be calculated in terms of oestrone and oestriol, curves showing the relationship between weights of the pure compounds and maximum intensities of the red colours had to be constructed. The oestrone curve was constructed from a sample of the International Oestrone Standard.

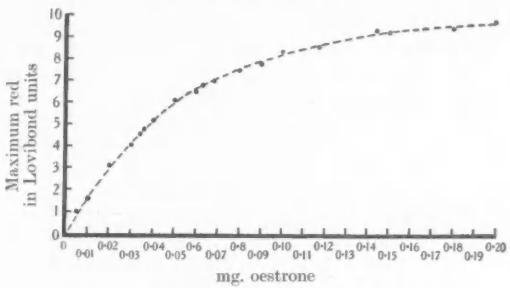



Fig. 6. Standardisation curve for oestrone.

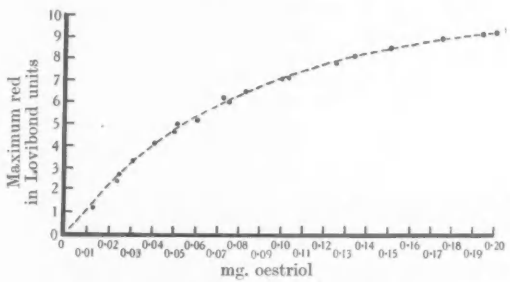



Fig. 7. Standardisation curve for oestriol.

For the oestriol curve, a highly purified specimen, prepared by Dr André Girard and very kindly presented to one of the authors, was used. Colours were developed by 2 ml. of reagent B and 5 % sulphuric acid as diluent. The standardisation curves are shown in Figs. 6 and 7. It will be seen that, as Kober pointed

out, the relationship between intensity of maximum red colour and quantity of hormone is not linear. Owing to the shape of the curves, the most accurate results are obtained from colours between 3 and 7 Lovibond red units.

(*d*) *Hydrolysis of the ether-insoluble "oestrin esters" in urine.* Zondek [1934] has recently stated that for human pregnancy urine (but not for pregnant mare's urine) no preliminary treatment with acid is required for the complete removal of the oestrin by extraction with immiscible solvents. The majority of other workers in the field believe, on the contrary, that the yields of oestrin obtainable from human urine can be very considerably increased by a preliminary treatment with acid [*cf.* Marrian, 1933; Borchardt *et al.*, 1934]. Hence it is generally assumed that much of the oestrin in urine is present as a water-soluble, ether-insoluble ester. Owing presumably to the great labour involved in carrying out large numbers of biological assays of oestrin, no systematic attempt has hitherto been made to determine the optimum conditions for the hydrolysis of these "oestrin esters" by acid. Furthermore, little attention has been paid in the past to the possibility of effecting the hydrolysis by other means than acids, although Marrian [1933] reported experiments in which alkaline hydrolysis at 100° was found to be ineffective, while allowing the urine to putrefy was found to be more effective than acid hydrolysis for one hour at 100°. Both these facts have been confirmed by the present authors.

Before this colorimetric method could be applied to the quantitative estimation of oestrin in urine, it was obviously necessary to determine the optimum conditions for the preliminary hydrolysis. Experiments have therefore been carried out in which urines have been hydrolysed under varying conditions for varying times before extraction and fractionation. In this way it has been possible to determine the rate of release from an ether-insoluble form, on hydrolysis under different conditions, of the chromogenic material which appeared in both the oestrone and oestriol fractions. As will be shown later, the assumption that all the chromogenic material released on hydrolysis was oestrogenic proved to be justified.

The results obtained by these means may conveniently be summarised at this point. It was found that on heating pregnancy urine at 100° after acidification with hydrochloric acid to $p_H$ 1–2, a rapid and almost linear increase of ether-soluble chromogen in both oestrone and oestriol fractions occurred for about 2 hours. From 2 to 16 hours the amount still increased but at a much slower rate. On heating for more than 16 hours, definite evidence of destruction of the hormone of both phenolic fractions was obtained. On incubating urine at 37°, a gradual increase of ether-soluble chromogens occurred. A maximum higher than that obtained by heating at 100° at $p_H$ 1–2, was arrived at after about 2 weeks' incubation. On further incubation, destruction of the chromogens took place. By heating the urine acidified with hydrochloric acid to about $p_H$ 1–2 in an autoclave at 15 lbs. for 2–4 hours, a degree of hydrolysis as great as the optimum for incubation at 37° was obtained. On more prolonged autoclaving, slow destruction occurred. Some of the values obtained for pregnancy urine (batch $PU_7$) are given in Table III to illustrate most of these points. Confirmatory results for the conclusions stated above were also obtained with other batches of urine.

It is as yet impossible to state definitely whether or not complete hydrolysis of the "oestrin esters" in urine has been effected by any of these methods. So far, however, no method of hydrolysis giving a yield higher than that given by autoclaving at $p_H$ 1–2 for 2–4 hours has been found. As a temporary working hypothesis, therefore, the assumption will be made that hydrolysis under these conditions is complete.

Table III. *Effect of hydrolysis of pregnancy urine on the oestrin content of ether extracts.*

| | | mg. oestrin per 100 ml. urine | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Acid-hydrolysed at 100° | | | Incubated at 37° | | Acid-autoclaved | |
| Urine batch | | Not hydrolysed | 2 hrs. | 16 hrs. | 48 hrs. | 2 weeks | 4 weeks | 1 hr. | 4 hrs. |
| $PU_2$ | Oestriol | 0·420 | 0·540 | 0·800 | 0·620 | 1·120 | 0·560 | 0·620 | 1·000 |
| | Oestrone | 0·024 | 0·046 | 0·070 | 0·065 | 0·080 | 0·047 | 0·070 | 0·082 |

(*e*) *The recovery of oestrone and oestriol added to mixtures.* The conditions necessary for the production of normal colour development curves from the phenolic fractions of pregnancy urine having been established, it was thought desirable to test the efficacy of the whole method by experiments on solutions containing known amounts of oestrone and oestriol. For this purpose solutions in 400 ml. of water or of male urine, containing known weights of oestriol and/or oestrone, and in one case also cholesterol and pregnandiol, were made up. These were acidified with hydrochloric acid, in some cases heated to 100° for 4 hours, and then ether-extracted. The ethereal extracts were washed with aqueous sodium carbonate solution and then subjected to the oestrone-oestriol separation process as shown in Table II. Colour tests on aliquot portions of the oestrone and oestriol fractions were then carried out using 2 ml. of the reagent. The results are shown in Table IV.

Table IV. *The recovery of oestrone and oestriol from mixtures of known composition.*

| | mg. added to 400 ml. water | | | | | mg. recovered | | % recovery | |
|---|---|---|---|---|---|---|---|---|---|
| | Oestriol | Oestrone | Cholesterol | Pregnandiol | Hydrolysis | Oestriol fraction | Oestrone fraction | Oestriol fraction | Oestrone fraction |
| I | 2·000 | — | — | — | None | 1·600 | 0·053 | 80 | — |
| II | — | 0·200 | — | — | ,, | 0·012 | 0·190 | — | 95 |
| III | 1·000 | 0·100 | — | — | ,, | 0·870 | 0·106 | 87 | 106 |
| IV | 1·000 | 0·100 | — | — | 4 hrs. HCl at 100° | 0·870 | 0·100 | 87 | 100 |
| V | 0·500 | 0·100 | — | — | ,, | 0·465 | 0·100 | 93 | 100 |
| VI | 1·500 | 0·400 | — | — | ,, | 1·250 | Fraction lost | 83 | — |
| VII | 1·000 | 0·200 | — | — | ,, | 0·780 | 0·192 | 78 | 96 |
| VIII | 1·000 | 0·100 | 1·0 | 1·0 | ,, | 0·890 | 0·106 | 89 | 106 |
| IX | 1·000 | 0·100 (in 400 ml. of male urine) | | | ,, | 0·965 | 0·093 | 97 | 93 |

It will be seen that there is an average loss of about 13 % of oestriol in these experiments, which is hardly surprising when the somewhat elaborate nature of the whole process is taken into consideration. At first sight it might appear that the recovery of the oestrone was nearly quantitative. It will be seen, however, that from the solution containing oestriol but no oestrone, a small amount of "oestrone" was "recovered." This is undoubtedly due to the fact that traces of oestriol had escaped extraction from the ethereal solution by the $N/10$ alkali and were therefore later extracted by the $N$ alkali and hence appeared in the oestrone fraction. It should be pointed out that since the mixtures contained approximately ten times more oestriol than oestrone—the proportion usually present in pregnancy urine—a negligible loss of oestriol would cause a not inconsiderable error in the oestrone figure. The oestrone-oestriol separation process is therefore not strictly quantitative. Nevertheless the figures obtained seem to justify the conclusion that for most practical purposes the separation can be

considered to be nearly quantitative. It is believed that in determinations on urines the error in the oestrone value should not be large, provided that abnormally high amounts of oestriol and low amounts of oestrone are not present.

(*f*) *Comparison of the colorimetric and biological methods of assay.* Finally it had to be shown that the chromogenic material obtained in the ether-soluble phenolic fractions of pregnancy urine was all oestrogenic. Comparisons of the biological and colorimetric values obtained for the oestrone and oestriol contents of fractions obtained from different samples of urine subjected to various types of preliminary hydrolysis were made.

The biological tests were carried out on ovariectomised mice by the method of Marrian and Parkes [1929]. Standardisation curves for the mouse colony for oestrone and oestriol were first constructed[1], using the same standard preparations as those used for the colorimetric standardisation curves.

Table V shows the values obtained. The close agreement between the figures obtained by the two methods of assay clearly shows that for human pregnancy urine the modified Kober test is specific for oestrone and oestriol.

Table V. *Comparison of the values for the oestrin content of urinary phenolic fractions obtained by the biological and colorimetric methods of assay.*

| | | Oestriol mg./100 ml. urine | | Oestrone mg./100 ml. urine | |
|---|---|---|---|---|---|
| Urine batch | Conditions of hydrolysis of the urine | Biological | Colorimetric | Biological | Colorimetric |
| P.U. 3 | 4 hrs.; acid at 100° | 0·243<br>0·231 | 0·205<br>0·240 | — | — |
| P.U. 4 | „ | 0·171<br>0·178 | 0·182<br>0·178 | 0·024<br>0·031 | 0·033<br>0·042 |
| P.U. 5 | 8 hrs.; acid at 100° | 0·265<br>0·218<br>0·224<br>0·231<br>— | 0·316<br>0·306<br>0·275<br>0·280<br>0·300 | 0·070<br>— | 0·071<br>0·072 |
| P.U. 6 | 16 hrs.; acid at 100° | 0·624<br>0·607<br>— | 0·691<br>0·700<br>0·630 | 0·107<br>— | 0·100<br>0·100 |
| P.U. 6 | 4 weeks; incubation | — | — | 0·126<br>0·115 | 0·127<br>0·100 |
| P.U. 7 | 16 hrs.; acid at 100° | 0·593<br>0·558 | 0·680<br>0·705 | — | — |
| P.U. 7 | 4 hrs.; acid-autoclaving | — | — | 0·092 | 0·100<br>0·077<br>0·077 |
| P.U. 8 | „ | 1·07 | 1·07<br>1·00<br>0·970<br>1·00 | 0·125<br>0·121<br>— | 0·127<br>0·123<br>0·107 |
| I.P.U., i, B | „ | 0·887 | 1·20<br>1·13 | — | — |
| I.P.U., i, C | „ | 1·22<br>0·989 | 0·800<br>0·875 | — | — |

[1] It was previously found by one of us (G. F. M.) that oestrone was slightly more than twice as active as oestriol when tested on ovariectomised mice. In the present work the potencies of oestrone and oestriol have been found to be 13,000 m.u. and 8130 m.u. per mg. respectively. It appears therefore that the strain of mice used in Toronto is relatively more sensitive to oestriol than were the English mice previously used.

## Discussion.

The authors are of the opinion that the evidence presented in this paper has shown that the oestrone and oestriol in human pregnancy urine may be separated and estimated with a reasonable degree of accuracy by means of a modified Kober colour reaction. The separation of the oestrone and oestriol, although not strictly quantitative, is nevertheless sufficiently complete to permit the detection of any abnormal amounts of either compound in pregnancy urine. The quantitative colour test *per se* is certainly as accurate as, if not more accurate than, the biological method of assay and can be carried out in a few minutes, whereas several days at least are required for the performance of a test by the latter method.

For the convenience of any other worker who may wish to use this method of assay, the exact procedure finally adopted is as follows:

(*a*) *Preparation of the phenolic fractions from urine.* A 300 ml.[1] sample of pregnancy urine is adjusted to $p_H$ 1–2 with hydrochloric acid and heated in an autoclave for 2–4 hours at a pressure of 15 lbs. After cooling, the hydrolysed urine is extracted once with 150 ml. and then three times with 100 ml. lots of freshly redistilled ether[2]. The combined ethereal extracts are then washed four times with 30 ml. lots of 10 % aqueous sodium carbonate solution, these washings being discarded. The "strong phenolic" fraction containing the oestriol is removed from the ethereal solution by extracting four times with 30 ml. volumes of $N/10$ sodium hydroxide. This alkaline extract is then washed three times with 30 ml. lots of ether, in order to remove any oestrone that may have been extracted.

The two ethereal solutions are combined, washed four times with 30 ml. lots of water and evaporated to dryness. The residue is dissolved in 100 ml. of toluene, and the "weak phenolic" fraction containing the oestrone is removed from the solution by four extractions with 30 ml. volumes of $N$ sodium hydroxide.

The $N/10$ and $N$ sodium hydroxide solutions containing respectively the oestriol and oestrone phenolic fractions are saturated with carbon dioxide until they are no longer alkaline to phenolphthalein and then extracted four times with 30 ml. lots of ether. The two ethereal solutions are finally washed four times with 25 ml. lots of water and evaporated to dryness. The residues containing the oestriol and oestrone fractions are then dissolved in alcohol, and aliquot samples of each solution are removed for the colorimetric assay.

(*b*) *The colorimetric assay on the phenolic fractions.* The sample for assay is measured into a test-tube and evaporated to dryness at 100° under a stream of nitrogen. To the residue are added 2·0 ml. of the phenolsulphonic acid reagent, the tube is heated for 10 minutes at 100° and then immediately cooled in a freezing mixture. The reaction mixture is transferred to a graduated test-tube (1 cm. diameter) with 5 % sulphuric acid and the volume made up to exactly 4 ml. The initial colour of the solution and the colours after heating at 100° for successive 30 second periods and cooling are analysed in the Lovibond tintometer. For the actual colour measurement, the solution is transferred to the standard 1 cm. tintometer cell. For subsequent heating and cooling, the solution

[1] All pregnancy urines so far tested contained sufficient oestrone and oestriol to enable the tests to be carried out with no more than 300 ml. This may not be the case with urines obtained in the early stages of pregnancy.

[2] The purity of the ether is of the utmost importance. In experiments in which pure oestriol was treated with impure ether, marked inhibition of colour development occurred. This was presumably due to the peroxide content of the ether.

is returned to the graduated test-tube. Colour measurements at 30-second intervals are continued until the maximum red value has been attained and passed. 0·5 ml. of 3 % hydrogen peroxide is then added and the solution heated at 100° for 2 minutes. The remaining colour of the solution, if any, is analysed in the tintometer and the value of the red component subtracted from the red maximum originally obtained, after making the necessary correction for the change in volume caused by the addition of the peroxide. The weight of oestrone or oestriol corresponding to the corrected red maximum is finally read from the appropriate standardisation curve. To ensure the greatest possible accuracy in the colorimetric determination, it is necessary to conduct several estimations on different amounts of the phenolic fractions.

The authors wish to express their deep gratitude to the Connaught Laboratories for generous contributions towards the expenses involved in carrying out the necessarily numerous biological assays.

They are also deeply indebted to the Toronto General Hospital (and in particular to Prof. W. B. Hendry and Dr J. Mann) for the supplies of pregnancy urine.

Finally they wish to express appreciation of the skilful assistance of Mr M. Edson in carrying out the biological assays.

## REFERENCES.

Borchardt, Dingemanse and Laqueur (1934). *Naturwiss.* **22**, 110.
Kober (1931). *Biochem. Z.* **239**, 209.
Marrian (1933). *Physiol. Rev.* **13**, 185.
—— and Parkes (1929). *J. Physiol.* **67**, 390.
Zondek (1934). *Nature*, **133**, 309.

# CCXIII. NON-DIFFUSIBLE SERUM-CALCIUM AT VARIOUS CALCIUM, PHOSPHATE AND HYDROGEN ION CONCENTRATIONS.

By RALPH GRAFTON SMITH.

*From the Department of Materia Medica and Therapeutics, University of Michigan Medical School, Ann Arbor, Michigan.*

(*Received May 11th, 1934.*)

The study of the diffusibility of blood serum-calcium has been an interesting development in the investigation of calcium metabolism. The finding of Rona and Takahashi [1911], that only a part of the serum-calcium was diffusible through membranes impermeable to protein, has been corroborated by many observers and by various methods. The figures for diffusibility recorded in the literature cover a wide range, but most of them lie between 45 and 70 % of the total serum-calcium. It is generally assumed that the non-diffusible fraction is bound to protein, this being suggested by the removal of a certain fraction of calcium in serum-protein precipitation [Csapo and Faubl, 1924], by the parallelism between the non-diffusible calcium and protein contents of various body fluids [Salvesen and Linder, 1923–24; Liu, 1927], by an apparent binding of a fraction of the calcium when $CaCl_2$ is added to protein solutions, as shown by diffusion experiments [Loeb, 1928; Marrack and Thacker, 1926], and by conductivity titrations when calcium is added to serum solutions [Shear and Offner, 1931]. Furthermore, when the total serum-calcium concentration is increased the non-diffusible fraction also increases [Loeb and Nichols, 1927; Brull, 1930; Smith and Sternberger, 1932; and Benjamin and Hess, 1933]. It is natural to assume that at least a part of this process is due to a further combination of calcium with serum-protein to preserve the equilibrium between protein-bound calcium and calcium ions.

In contrast to the state of serum-calcium, the inorganic phosphate of mammalian serum is generally agreed to be entirely diffusible or almost so [Rona and Takahashi, 1913; Cushny, 1919–20; Neuhausen and Pincus, 1923; Grollman, 1927; Brull, 1930; Greene and Power, 1931; Benjamin and Hess, 1933]. However, the interesting observation was made by Grollman [1927] by ultrafiltration of pig and dog sera that inorganic phosphate gradually becomes non-diffusible as the serum-calcium is raised above the normal level. He suggested the formation of a colloidal complex of $Ca_3(PO_4)_2$ and protein. This finding has since been corroborated by other observers [Brull, 1930; Benjamin and Hess, 1933; Laskowski, 1933].

Thus when calcium is added to serum a part of the added calcium becomes non-diffusible. As Laskowski [1933] has stated, this may be accounted for by two different processes—firstly by a combination of calcium with protein (as occurs in phosphate-free protein solutions [Loeb, 1928]), and secondly by the formation of a calcium phosphate complex with protein (as suggested by the formation of non-diffusible phosphate along with the increase in non-diffusible

calcium). The question naturally arises: Do the two processes occur simultaneously, or is the newly formed non-diffusible calcium at first the result of calcium phosphate formation only until all the free phosphate has been combined in this manner, with a secondary binding of calcium by protein if the calcium concentration is sufficiently increased?

The present paper reports a series of *in vitro* ultrafiltration experiments which indicates the simultaneous occurrence of both processes. Also more direct evidence is presented for the formation of $Ca_3(PO_4)_2$. Furthermore, by carrying out experiments in which the effect of changes in hydrogen ion concentration on the diffusibility of serum-calcium was studied, the importance of the phosphate factor in such variations is indicated.

## Experimental methods.

Blood was obtained from normal dogs by exsanguination under light ether anaesthesia and allowed to clot at room temperature. The serum was separated by centrifuging and was stored in stoppered tubes in the refrigerator, the experiments being carried out within 24 hours after obtaining the blood. The calcium and phosphate concentrations were increased as desired by the addition of small volumes of 10 % $CaCl_2$ and $Na_2HPO_4$ solutions. The latter was brought to $p_H$ 7·4 with HCl. Variations in hydrogen ion concentration were made by adding 0·25 ml. of NaOH or HCl of suitable strengths to 10 ml. samples of serum. An equivalent volume of saline was added to the control sample in each experiment. Such additions increased the volume of the normal serum by from 1 to 5 %.

5 ml. samples of serum were ultrafiltered through collodion sacs impermeable to protein at 150 mm. Hg pressure by the method of Greenberg and Gunther [1929–30] with minor modifications as previously described [Smith and Sternberger, 1932]. Calcium was determined by the Clark-Collip [1925] modification of the Kramer-Tisdall method, allowing at least 2 hours for the precipitation of calcium oxalate, inorganic phosphate by the method of Fiske and Subbarow [1925], and $p_H$ by the colorimetric method of Hastings and Sendroy [1924].

Hydrogen ion concentration experiments were unsatisfactory to some degree in that the $p_H$ of the residue and usually of the ultrafiltrate rose above that of the original serum during ultrafiltration, presumably owing to a loss of $CO_2$. This was not avoided by connecting the collodion sacs to flasks containing $NaHCO_3$ in buffer solutions of the desired $p_H$ as suggested by Greenberg and Gunther [1929–30]. The $p_H$ readings recorded are those of the serum at the beginning of ultrafiltration.

## Results.

Calcium and inorganic phosphate concentrations are expressed in mg. per 100 ml. of serum. The diffusible calcium and phosphate were calculated from the ultrafiltrate concentration by making a 5 % correction for protein volume in all experiments as previously described [Smith and Sternberger, 1932].

### *Variations in calcium and phosphate concentrations.*

In Table I are recorded the results of experiments in which the calcium concentration has been gradually increased in separate samples of the same serum. The "increase in non-diffusible Ca and P" (columns (8) and (9)) refers to the increases of these fractions above the level present in the normal serum which is recorded in the first line in each experiment. The Ca : P ratio of these values

Table I. *Effect of increased calcium concentration on diffusibility of serum-calcium and inorganic phosphate.*

| | Serum-Ca | | | Serum-inorganic $PO_4$ (P) | | | Increase in non-diffusible | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Exp. No. | Total mg. | Diffusible mg. | Non-diffusible mg. | Total mg. | Diffusible mg. | Non-diffusible mg. | Ca mg. | P mg. | Ca: P | $p_H$ |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) |
| I | 10·0 | 6·2 | 3·8 | 5·2 | 5·4 | — | — | — | — | 7·82 |
| | 22·4 | 12·2 | 10·2 | 5·2 | 3·4 | 1·8 | 6·4 | 1·8 | 3·6 | 7·85 |
| | 35·0 | 18·7 | 16·3 | 5·2 | 1·7 | 3·5 | 12·5 | 3·5 | 3·6 | 7·85 |
| II | 9·7 | 5·5 | 4·2 | 5·6 | 5·7 | — | — | — | — | 7·75 |
| | 20·9 | 12·0 | 8·9 | 5·6 | 4·2 | 1·4 | 4·7 | 1·4 | 3·4 | 7·85 |
| | 31·0 | 17·6 | 13·4 | 5·6 | 3·0 | 2·6 | 9·2 | 2·6 | 3·5 | 7·80 |
| III | 11·2 | 6·5 | 4·7 | 11·6 | 11·6 | — | — | — | — | — |
| | 22·8 | 10·5 | 12·3 | 11·6 | 7·6 | 4·0 | 7·6 | 4·0 | 1·9 | — |
| IV | 9·7 | 5·6 | 4·1 | 5·2 | 5·2 | — | — | — | — | — |
| | 20·8 | 10·1 | 10·7 | 5·2 | 3·1 | 2·1 | 6·6 | 2·1 | 3·1 | — |
| | 31·8 | 16·9 | 14·9 | 5·2 | 2·3 | 2·9 | 10·8 | 2·9 | 3·7 | — |
| V | 10·1 | 6·5 | 3·6 | 7·0 | 7·1 | — | — | — | — | 7·57 |
| | 22·3 | 13·9 | 8·4 | 7·0 | 5·5 | 1·5 | 4·8 | 1·5 | 3·2 | 7·53 |
| | 33·0 | 20·7 | 12·3 | 7·0 | 4·1 | 2·9 | 8·7 | 2·9 | 3·0 | 7·63 |
| VI | 10·7 | 5·9 | 4·8 | 5·7 | 5·8 | — | — | — | — | 7·77 |
| | 16·2 | 7·9 | 8·3 | 5·7 | 4·6 | 1·1 | 3·5 | 1·1 | 3·2 | 7·77 |
| | 24·0 | 12·6 | 11·4 | 5·7 | 3·6 | 2·1 | 6·6 | 2·1 | 3·1 | 7·80 |
| | 29·6 | 14·3 | 15·3 | 5·7 | 2·2 | 3·5 | 10·5 | 3·5 | 3·0 | 7·82 |
| | 35·4 | 17·1 | 18·3 | 5·7 | 1·9 | 3·8 | 13·5 | 3·8 | 3·6 | 7·85 |
| VII | 10·8 | 6·9 | 3·9 | 9·5 | 8·8 | 0·7 | — | — | — | — |
| | 69·0 | 49·0 | 20·0 | 9·5 | 1·6 | 7·9 | 16·1 | 7·2 | 2·2 | — |
| | 143·0 | 117·0 | 26·0 | 9·5 | 0·7 | 8·8 | 22·1 | 8·1 | 2·7 | — |
| VIII | 10·4 | 6·0 | 4·4 | 7·3 | 7·7 | — | — | — | — | 7·77 |
| | 46·0 | 23·5 | 22·5 | 7·3 | 2·1 | 5·2 | 18·1 | 5·2 | 3·5 | 7·77 |
| | 118·0 | 90·0 | 28·0 | 7·3 | 0·9 | 6·4 | 23·6 | 6·4 | 3·7 | 7·77 |
| IX | 9·0 | 6·0 | 3·0 | 5·7 | 6·3 | — | — | — | — | 7·72 |
| | 26·5 | 15·2 | 11·3 | 5·7 | 3·1 | 2·6 | 8·3 | 2·6 | 3·2 | 7·77 |
| | 43·5 | 29·7 | 13·8 | 5·7 | 2·1 | 3·6 | 10·8 | 3·6 | 3·0 | 7·80 |
| | 79·5 | 58·6 | 20·9 | 5·7 | 0·8 | 4·9 | 17·9 | 4·9 | 3·6 | 7·80 |
| | 127·0 | 102·0 | 25·0 | 5·7 | 0·4 | 5·3 | 22·0 | 5·3 | 4·1 | 7·75 |

(with one exception which will be mentioned later) is higher than the ratio in such calcium phosphates as $CaHPO_4$ (Ca: P = 1·3) or $Ca_3(PO_4)_2$ (Ca: P = 1·9). This of course is to be expected, if a combination with protein is responsible for a part of the increase in non-diffusible calcium. The results show further that the two processes occur simultaneously, *i.e.* the phosphate gradually becomes non-diffusible as the calcium concentration is increased, suggesting a combination of calcium and phosphate, yet the Ca: P ratio of the increase in non-diffusible compounds remains between 3 and 4 throughout, indicating, both with small and large increases in calcium concentration, some other combination—presumably with protein. In most cases there is some increase in the ratio with higher calcium concentrations, but even above 100 mg. per 100 ml. as in Exps. VII, VIII and IX this increase is not marked. Thus it is evident that when the greater part of the phosphate has become combined, the calcium-combining power of the protein is also approaching exhaustion.

In the results of Table I there is nothing to suggest the type of calcium phosphate formed apart from its non-diffusibility. However, in Table II which records similar experiments carried out in the presence of excess phosphate

Table II. *Effect of increased calcium concentration on diffusibility of serum-calcium and inorganic phosphate in the presence of excess phosphate.*

| | Serum-Ca | | | Serum-inorganic $PO_4$ (P) | | | Increase in non-diffusible | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Exp. No. | Total mg. | Diffusible mg. | Non-diffusible mg. | Total mg. | Diffusible mg. | Non-diffusible mg. | Ca mg. | P mg. | Ca : P | $p_H$ |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) |
| I | 10·0 | 7·0 | 3·0 | 7·7 | 8·0 | — | — | — | — | 7·77 |
| | 33·6 | 7·2 | 26·4 | 30·8 | 19·1 | 11·7 | 23·4 | 11·7 | 2·0 | 7·92 |
| II | 9·8 | 5·8 | 4·0 | 7·6 | 7·9 | — | — | — | — | 7·73 |
| | 40·7 | 7·6 | 33·1 | 30·5 | 15·5 | 15·0 | 29·1 | 15·0 | 1·9 | 7·90 |
| III | 9·6 | 6·0 | 3·6 | 5·7 | 5·7 | — | — | — | — | 7·72 |
| | 38·0 | 8·7 | 29·3 | 26·0 | 12·6 | 13·4 | 25·7 | 13·4 | 1·9 | 7·88 |
| IV | 10·0 | 5·9 | 4·1 | 5·4 | 5·6 | — | — | — | — | 7·60 |
| | 22·0 | 7·2 | 14·8 | 15·4 | 10·2 | 5·2 | 10·7 | 5·2 | 2·1 | 7·65 |
| | 36·7 | 7·7 | 29·0 | 25·0 | 13·4 | 11·6 | 24·9 | 11·6 | 2·1 | 7·77 |
| V | 9·9 | 5·3 | 4·6 | 6·3 | 6·4 | — | — | — | — | 7·67 |
| | 35·1 | 5·1 | 30·0 | 38·0 | 24·1 | 13·9 | 25·4 | 13·9 | 1·8 | 7·67 |
| | 58·8 | 9·8 | 49·0 | 38·0 | 14·8 | 23·2 | 44·4 | 23·2 | 1·9 | 7·72 |
| | 85·0 | 21·5 | 63·5 | 38·0 | 7·3 | 30·7 | 58·9 | 30·7 | 1·9 | 7·77 |
| VI | 9·8 | 5·4 | 4·4 | 4·8 | 4·8 | — | — | — | — | 7·47 |
| | 22·8 | 9·0 | 13·8 | 19·4 | 14·4 | 5·0 | 9·4 | 5·0 | 1·9 | 7·50 |
| | 34·2 | 10·3 | 23·9 | 19·4 | 9·9 | 9·5 | 19·5 | 9·5 | 2·0 | 7·50 |
| | 57·4 | 21·1 | 36·3 | 19·4 | 4·7 | 14·7 | 31·9 | 14·7 | 2·2 | 7·50 |
| | 92·4 | 41·5 | 50·9 | 19·4 | 2·8 | 16·6 | 46·5 | 16·6 | 2·8 | 7·48 |
| VII | 9·1 | 5·7 | 3·4 | 4·6 | 4·6 | — | — | — | — | 7·52 |
| | 20·9 | 9·3 | 11·6 | 18·3 | 14·1 | 4·2 | 8·2 | 4·2 | 2·0 | 7·52 |
| | 45·8 | 16·8 | 29·0 | 18·3 | 7·5 | 10·8 | 25·6 | 10·8 | 2·4 | 7·50 |
| | 68·8 | 31·9 | 36·9 | 18·3 | 4·5 | 13·8 | 33·5 | 13·8 | 2·4 | 7·53 |
| | 114·0 | 71·2 | 42·8 | 18·3 | 2·1 | 16·1 | 39·4 | 16·1 | 2·4 | 7·55 |

the Ca: P ratio of the newly formed non-diffusible compounds is 1·9 or approximates to that value very closely, *i.e.* to the ratio of these elements in $Ca_3(PO_4)_2$. Some variation in this result may be expected in that it involves the combined errors of two calcium and two phosphate determinations and of the ultrafiltration process. In spite of this fact variations from 1·9, which occur in certain cases, are comparatively slight. This ratio is constant, irrespective of the amount of calcium and phosphate added, provided that the free phosphate remains above the level in the normal serum. However, as calcium is added in great excess, as in Exps. VI and VII, the diffusible phosphate falls below the level usually present in the normal serum and the ratio rises, in Exp. VI to 2·4, in Exp. VII to 2·8. Apparently in the presence of excess phosphate calcium combines only with phosphate to form $Ca_3(PO_4)_2$ which of course, in turn, may form a colloidal complex with protein as previously suggested. After the excess phosphate has been combined, further additions of calcium combine also with protein as indicated by the rise of the Ca : P ratio above 1·9. Of course such results do not prove that the normal serum-inorganic phosphate forms $Ca_3(PO_4)_2$ with added calcium but they suggest this possibility. Further evidence for it is found in Exp. III of Table I in which the serum-inorganic phosphate of this particular dog was definitely higher than that usually found. In this case the added calcium was sufficient to depress the diffusible phosphate only from 11·6 to 7·6 mg. per 100 ml., the latter figure still being a moderately high normal value. The Ca : P ratio of the increase in non-diffusible fractions was 1·9 as in those experiments in which phosphate was actually added to the serum.

*Variations in hydrogen ion concentration.*

It is known that in normal serum wide variations in $p_H$ cause a great change in the diffusibility of calcium [Loeb, 1928] (see also Table III, Exps. I and II). With regard to changes in the physiological range Greenberg and Gunther [1929–30] have reported that no change in diffusibility occurs between $p_H$ 7 and 8. Similarly Meysenbug *et al.* [1921] found no change betwen $p_H$ 7·0 and 7·6. More recently Dillman and Visscher [1933] have reviewed the literature on this subject and have reported results contrary to the above findings. The serum-calcium diffusibility at $p_H$ 7·0 (59 %) differed quite markedly from that at $p_H$ 7·6 (45 %). Table III records the results of a series of ultrafiltration experi-

Table III. *Effect of hydrogen ion concentration on diffusibility of normal serum-calcium.*

| Exp. No. | Total Ca mg. | Diffusible Ca % | $p_H$ | Exp. No. | Total Ca mg. | Diffusible Ca % | $p_H$ |
|---|---|---|---|---|---|---|---|
| I | 9·1 | 17·5 | 10·1 | V | 9·5 | 51 | 8·0 |
| | | 54 | 7·6 | | | 55 | 7·9 |
| | | 106 | 4·1 | | | 55 | 7·75 |
| II | 8·4 | 16 | 10·2 | | | 56·5 | 7·55 |
| | | 26 | 9·2 | | | 55 | 7·35 |
| | | 54 | 7·7 | | | 57 | 7·15 |
| | | 68 | 7·0 | VI | 10·05 | 61·5 | 8·15 |
| | | 72 | 5·25 | | | 63·5 | 7·85 |
| | | 85 | 5·1 | | | 62 | 7·75 |
| III | 10·0 | 59 | 8·0 | | | 62 | 7·55 |
| | | 62 | 7·8 | | | 64 | 7·25 |
| | | 68·5 | 7·5 | | | 66 | 7·0 |
| | | 68 | 7·4 | VII | 9·15 | 54 | 7·95 |
| | | 65 | 7·2 | | | 51 | 7·77 |
| IV | 9·95 | 64 | 7·95 | | | 59 | 7·60 |
| | | 64 | 7·8 | | | 64 | 7·35 |
| | | 65 | 7·6 | | | 61 | 7·20 |
| | | 71 | 7·3 | | | 66 | 7·05 |
| | | 69 | 7·15 | | | | |
| | | 69 | 7·05 | | | | |

Exps. III–VII. Average maximum change in $p_H = 0·92$.
Average change in calcium diffusibility = 6·7 %.

ments on normal sera, showing some change in diffusibility between $p_H$ 7 and 8. With slight variations in $p_H$ the differences are well within the limits of experimental error, but the general direction of the change is the same in all experiments. On averaging the results of Exps. III–VII it is found that on decreasing the $p_H$ by 1·0 in this range, approximately 7 % more of the total calcium becomes diffusible. Thus the finding of Dillman and Visscher is confirmed although smaller changes in diffusibility are observed in the present experiments.

It was conceivable that the part played by calcium phosphate in the formation of non-diffusible calcium in hypercalcaemia might also be a factor in calcium diffusibility changes with variations in hydrogen ion concentration. In titration curves of $H_3PO_4$ with $Ca(OH)_2$, presented by Holt *et al.* [1925], $Ca_3(PO_4)_2$ was precipitated at $p_H$ 5–6. The point of precipitation was definitely changed by the presence of other ions, occurring at a slightly higher $p_H$ when the titration was carried out with $Ca(OH)_2$ in 0·1 *M* NaCl, and at $p_H$ 6·5–7·5 with $Ca(OH)_2$ in 0·1 *M* $MgSO_4$. In blood-serum, $Ca_3(PO_4)_2$ precipitation might

be expected to occur in this range, *i.e.* at a $p_H$ definitely higher than that of the isoelectric point of serum-protein, below which protein-bound calcium is freed [Loeb, 1928]. Thus, variations in $p_H$ in the former range would be expected to have a much greater effect on the diffusibility of calcium in hypercalcaemia if a considerable part of the non-diffusible calcium were due to phosphate, than in normal serum where the non-diffusible calcium is bound only to protein. This supposition was tested experimentally, the results being recorded in Table IV. Here, as predicted, calcium diffusibility is affected to a much greater

Table IV. *Effect of hydrogen ion concentration on calcium diffusibility in high calcium sera.*

| Total Ca and total $PO_4$ (P) mg. | Calcium | | Inorganic $PO_4$ (P) | | Non-diffusible Ca as | | |
|---|---|---|---|---|---|---|---|
| | Diffusible % | Non-diffusible mg. | Diffusible % | Non-diffusible mg. | $Ca_3(PO_4)_2$ mg. | Protein-bound mg. | $p_H$ |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) |
| Ca 23·2 | 57 | 10·0 | 62·5 | 1·2 | 2·3 | 7·7 | 8·05 |
| P 3·2 | 58 | 9·8 | 59·5 | 1·3 | 2·5 | 7·3 | 7·98 |
| | 58·5 | 9·6 | 65·5 | 1·1 | 2·1 | 7·5 | 7·88 |
| | 59·5 | 9·4 | 69 | 1·0 | 1·9 | 7·5 | 7·72 |
| | 64·5 | 8·2 | 101 | — | 0 | 8·2 | 7·52 |
| | 65·5 | 8·0 | 103 | — | 0 | 8·0 | 7·20 |
| Ca 23·7 | 47 | 12·5 | 42 | 2·9 | 5·5 | 7·0 | 8·05 |
| P 5·0 | 45 | 13·0 | 46 | 2·7 | 5·1 | 7·9 | 7·95 |
| | 52·5 | 11·2 | 60 | 2·0 | 3·8 | 7·4 | 7·75 |
| | 57·5 | 10·1 | 64 | 1·8 | 3·4 | 6·7 | 7·62 |
| | 63·5 | 8·7 | 90 | 0·5 | 0·9 | 7·8 | 7·05 |
| Ca 28·3 | 42 | 16·4 | 45 | 4·0 | 7·6 | 8·8 | 8·05 |
| P 7·3 | 42 | 16·3 | 45 | 4·0 | 7·6 | 8·7 | 7·92 |
| | 51 | 13·8 | 63 | 2·7 | 5·1 | 8·7 | 7·73 |
| | 49 | 14·5 | 57·5 | 3·1 | 5·9 | 8·6 | 7·55 |
| | 60 | 11·4 | 86·5 | 1·0 | 1·9 | 9·5 | 7·42 |
| | 64 | 10·1 | 87·5 | 0·9 | 1·7 | 8·4 | 7·25 |
| Ca 26·7 | 51 | 13·1 | 46·5 | 3·1 | 5·9 | 7·2 | 8·10 |
| P 5·8 | 53 | 12·5 | 51·5 | 2·8 | 5·3 | 7·2 | 7·70 |
| | 61 | 10·4 | 72·5 | 1·6 | 3·0 | 7·4 | 7·30 |
| | 71 | 7·7 | 99 | 0·05 | 0·1 | 7·6 | 6·88 |
| Ca 37·0 | 48·5 | 19·0 | 28 | 2·6 | 4·9 | 14·1 | 8·20 |
| P 3·6 | 50 | 18·4 | 28 | 2·6 | 4·9 | 13·5 | 7·85 |
| | 54 | 16·9 | 36 | 2·3 | 4·4 | 12·5 | 7·48 |
| | 59 | 15·2 | 69·5 | 1·1 | 2·1 | 13·1 | 7·05 |
| Ca 31·4 | 55·5 | 14·0 | 39 | 2·7 | 5·1 | 8·9 | 8·10 |
| P 4·4 | 61·5 | 12·1 | 54·5 | 2·0 | 3·8 | 8·3 | 7·75 |
| | 64·5 | 11·2 | 61 | 1·7 | 3·2 | 8·0 | 7·35 |
| | 71 | 9·1 | 75 | 1·1 | 2·1 | 7·0 | 7·00 |
| | 79 | 6·7 | 98 | 0·1 | 0·2 | 6·5 | 6·60 |

Average maximum change in $p_H = 1{\cdot}11$.
Average change in calcium diffusibility = 16·8 %.

extent by such changes in $p_H$. By averaging the changes in the six experiments it is found that approximately 15 % more of the total calcium becomes diffusible with a decrease in $p_H$ of 1·0 in this range. In a general way the magnitude of the change varies directly with the inorganic phosphate concentration and proceeds parallel with the liberation of the non-diffusible phosphate. In column (6) the non-diffusible calcium presumably present as $Ca_3(PO_4)_2$ is the product—non-diffusible phosphate × 1·9. That present in combination with protein, column (7), is the difference between $Ca_3(PO_4)_2$-calcium and the total non-diffusible calcium.

Such calculations show that the increase in calcium diffusibility with decrease in $p_H$ may be explained entirely by the disappearance of $Ca_3(PO_4)_2$. The protein-bound calcium shows little change, unlike its behaviour in normal serum. It is conceivable that any decrease with lowered $p_H$ would be counteracted by the greater concentration of diffusible calcium freed from $Ca_3(PO_4)_2$. The variations in column (7), with the possible exception of the last experiment, are not considered significant, as the values involve the errors of two calcium and two phosphate determinations as well as the ultrafiltration process.

If in the presence of excess phosphate the non-diffusible calcium, formed by the addition of calcium to serum, is due only to the formation of $Ca_3(PO_4)_2$, as indicated in Table II, similar changes in $p_H$ under such conditions should have a still greater effect. That such is the case is evident from Table V. Approxi-

Table V. *Effect of hydrogen ion concentration on calcium diffusibility in high calcium serums in the presence of excess phosphate.*

| Total Ca and total $PO_4$ (P) mg. | Calcium | | Inorganic $PO_4$ (P) | | Non-diffusible Ca as | | |
|---|---|---|---|---|---|---|---|
| | Diffusible % | Non-diffusible mg. | Diffusible % | Non-diffusible mg. | $Ca_3(PO_4)_2$ mg. | Protein-bound mg. | $p_H$ |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) |
| Ca 20·8 | 26 | 15·4 | 70 | 6·6 | 12·5 | 2·9 | 8·35 |
| P 22·0 | 26 | 15·4 | 71 | 6·4 | 12·1 | 3·3 | 8·23 |
| | 36·5 | 13·2 | 76·5 | 5·2 | 9·9 | 3·3 | 8·05 |
| | 51 | 10·2 | 88·5 | 2·5 | 4·7 | 5·5 | 7·72 |
| Ca 28·0 | 30 | 19·5 | 50 | 9·4 | 17·8 | 1·7 | 7·62 |
| P 23·3 | 32 | 19·0 | 63·5 | 8·5 | 16·1 | 2·9 | 7·28 |
| | 41·5 | 16·4 | 68·5 | 7·3 | 13·8 | 2·6 | 7·02 |
| | 69 | 8·7 | 90 | 2·3 | 4·4 | 4·3 | 6·68 |
| Ca 31·3 | 26 | 23·2 | 49 | 10·1 | 19·2 | 4·0 | 8·10 |
| P 19·7 | 30·5 | 21·7 | 49 | 10·1 | 19·2 | 2·5 | 7·80 |
| | 31·5 | 21·5 | 52 | 9·4 | 17·8 | 3·7 | 7·45 |
| | 49 | 16·1 | 70·5 | 5·8 | 11·0 | 5·1 | 6·90 |
| Ca 37·2 | 19 | 30·0 | 36·5 | 14·2 | 27·0 | 3·0 | 8·10 |
| P 22·4 | 27 | 27·3 | 44 | 12·5 | 23·8 | 3·5 | 7·85 |
| | 33 | 25·0 | 50 | 11·2 | 21·3 | 3·7 | 7·45 |
| | 46·5 | 19·9 | 63·5 | 8·2 | 15·6 | 4·3 | 7·03 |
| | 71 | 10·7 | 86 | 3·1 | 5·9 | 4·8 | 6·60 |

Average maximum change in $p_H = 1{\cdot}07$.
Average change in calcium diffusibility = 34·7 %.

mately 32 % more of the total calcium becomes diffusible by a decrease in $p_H$ of 1·0 in the 7–8 range, in contrast with 7 % with normal sera and 15 % in hypercalcaemia without added phosphate. Here also (Table V) the change in calcium diffusibility is entirely accounted for by the liberation of $Ca_3(PO_4)_2$. In fact the protein-bound calcium even increases slightly in spite of the decrease in $p_H$, which is readily accounted for by the increased concentration of diffusible calcium as it is freed from $Ca_3(PO_4)_2$.

## Discussion.

Brull [1930] by *in vivo* ultrafiltration showed that when serum-calcium is increased the non-diffusible Ca increases to a much greater extent than the non-diffusible phosphate, in contrast with the results when phosphate alone,

or when both calcium and phosphate, are increased, in which case the non-diffusible calcium and phosphate increase in parallel. This was presented as evidence that the greater part of the serum-inorganic phosphate existed in a non-ionised state. In the light of the present work this postulation is unnecessary. When the serum-calcium is increased non-diffusible calcium increases to a much greater extent than the non-diffusible phosphate, as only a part of it combines with phosphate, the remainder being bound directly by protein. However, when both calcium and phosphate are added the calcium reacts only with the phosphate, provided an excess is present; thus the non-diffusible fraction of each increases in close parallel. If sufficient calcium is added, approximately all the normal serum-phosphate becomes non-diffusible, and there is nothing to indicate that it behaves differently from added phosphate.

Greenberg and Gunther [1929–30] have expressed the protein-bound calcium as a function of the protein and ionic calcium concentrations. It must be recognised that this applies only to protein-bound calcium and not necessarily to the total non-diffusible calcium. As these authors state, according to the equation presented, even when the ionic calcium approaches infinity the protein-bound calcium becomes only of the same order as the total calcium of blood-serum. In a number of experiments of the present study the non-diffusible calcium rises much beyond this level, explicable, of course, by the fact that a considerable part of it is due to calcium phosphate formation.

## Summary.

1. When non-diffusible serum-calcium is increased by the addition of calcium to serum, the two processes accountable for this action (a binding of added calcium by protein and the formation of calcium phosphate which may in turn form a colloidal complex with protein) occur simultaneously.

2. Further evidence is presented for the formation of $Ca_3(PO_4)_2$, in that, in the presence of excess phosphate the increase in non-diffusible calcium is wholly explicable by the formation of this compound.

3. A change in $p_H$ from 8 to 7 causes a slight increase in the diffusibility of normal serum-calcium. With increased calcium concentrations this increase is much greater, as a considerable part of the non-diffusible calcium fraction exists as $Ca_3(PO_4)_2$ which breaks down over this range of $p_H$.

## REFERENCES.

Benjamin and Hess (1933). *J. Biol. Chem.* **100**, 27.
Brull (1930). *Arch. Int. Physiol.* **32**, 138.
Clark and Collip (1925). *J. Biol. Chem.* **63**, 461.
Csapo and Faubl (1924). *Biochem. Z.* **150**, 509.
Cushny (1919–20). *J. Physiol.* **53**, 391.
Dillman and Visscher (1933). *J. Biol. Chem.* **103**, 791.
Fiske and Subbarow (1925). *J. Biol. Chem.* **66**, 375.
Greenberg and Gunther (1929–30). *J. Biol. Chem.* **85**, 491.
Greene and Power (1931). *J. Biol. Chem.* **91**, 183.
Grollman (1927). *J. Biol. Chem.* **72**, 565.
Hastings and Sendroy, Jr. (1924). *J. Biol. Chem.* **61**, 695.
Holt, La Mer and Chown (1925). *J. Biol. Chem.* **64**, 509.

Laskowski (1933). *Biochem. Z.* **265**, 401.
Liu (1927). *Chinese J. Physiol.* **1**, 331.
Loeb (1928). *J. Gen. Physiol.* **8**, 451.
—— and Nichols (1927). *J. Biol. Chem.* **72**, 687.
Marrack and Thacker (1926). *Biochem. J.* **20**, 580.
Meysenbug, Pappenheimer, Zucker and Murray (1921). *J. Biol. Chem.* **47**, 529.
Neuhausen and Pincus (1923). *J. Biol. Chem.* **57**, 99.
Rona and Takahashi (1911). *Biochem. Z.* **31**, 336.
—— —— (1913). *Biochem. Z.* **49**, 370.
Salvesen and Linder (1923–24). *J. Biol. Chem.* **58**, 617.
Shear and Offner (1931). *J. Biol. Chem.* **91**, 291.
Smith and Sternberger (1932). *J. Biol. Chem.* **96**, 245.